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Mestrado em Neurociências

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RESUMO

Estudar formas simples de aprendizagem, como a aprendizagem associativa, torna-se numa abordagem útil quando se pretende investigar o papel de certos mecanismos de plasticidade subjacentes a estes comportamentos.

O condicionamento clássico é uma forma de aprendizagem associativa que pode ser estudada através da capacidade dos ratinhos de condicionarem o reflexo de piscarem o olho, em função de um estimulo inicialmente neutro (Boele et al. 2010; Kandel et al. 2000). Experiências anteriores mostraram que este comportamento depende da função de um cerebelo intacto (Chen et al. 1996; McCormick et al. 1981; McCormick & Thompson 1984). Desde esta descoberta que os substratos neuronais deste comportamento têm vindo a ser estudados extensivamente. Algumas sinapses dentro do circuito cerebelar foram já propostas como estando associadas a componentes específicos do comportamento em estudo (Carey & Lisberger 2002). Os endocanabinóides, que são neuromoduladores retrógrados presentes em todo o cérebro (El Manira & Kyriakatos 2010), foram recentemente implicados em diferentes formas de plasticidade no cerebelo, em particular os receptores endocanabinóides de tipo 1 (CB1) (Carey et al. 2011). Estes receptores parecem ser ainda necessários para um desempenho normal dos ratinhos, em experiências de condicionamento (Kishimoto & Kano 2006). No presente trabalho experimental, cuja motivação advém das descobertas acima descritas, pretendia-se estudar quais os componentes do comportamento em estudo– como a percentagem, amplitude e temporização da resposta condicionada – que estavam a ser modulados pela sinalização dos endocanabinóides, e em que consistia essa modulação. Pretendia-se ainda estudar, especificamente em que células do circuito cerebelar é que os receptores CB1 estariam a exercer os seus efeitos na aprendizagem condicionada deste

comportamento. Os resultados obtidos mostram que em ratinhos que não expressam receptores CB1, globalmente ou especificamente nas células granulares, o desempenho nas experiências comportamentais de condicionamento encontra-se comprometido, tanto em termos de percentagem como em termos de amplitude das respostas condicionadas. Estes efeitos apresentam uma maior magnitude em ratinhos que não expressam os os receptores CB1 globalmente. Por outro lado, não foram observadas quaisquer diferenças em termos da correcta temporização das respostas condicionadas, em ratinhos que não expressavam receptores CB1.

Palavras-Chave: Condicionamento clássico; Cerebelo; Endocanabinóides; CB1R; Plasticidade

ABSTRACT

Studying simple forms of learning, such as conditioned behaviors, is a useful approach to investigate the role of the plasticity mechanisms that underlie these processes.

Classical conditioning of the eyelid is a form of associative learning that is based on the ability of mice to condition their eyelid responses to an initially neutral stimulus (Boele et al. 2010; Kandel et al. 2000).

This behavior has been described to relay on an intact cerebellum (Chen et al. 1996; McCormick et al. 1981; McCormick & Thompson 1984), and its neural correlates have been studied extensively ever since. To date, some synapses within the cerebellar circuit have already been linked to specific features of the eyeblink conditioning (Carey & Lisberger 2002). Endocannabinoids - retrograde neuromodulators, that are present throughout the brain (El Manira & Kyriakatos 2010) – have recently been implicated in different forms of plasticity at the cerebellum, particularly type 1 endocannabinoid receptors (CB1R) (Carey et al. 2011). These receptors are also required for a normal performance of the cerebellum-dependent eyeblink conditioning behavior (Kishimoto & Kano 2006). In the present research work, which was motivated by the findings described above, we tried to understand which features of the eyeblink conditioning – such as the percentage, amplitude, and timing of the conditioned response - were being modulated by CB1R signaling and in what way. Additionally we wanted to understand specifically in which cell types were these receptors exerting their effects in eyeblink conditioning. The results show that mice lacking CB1R's, both globally and in granule cells, have an impaired performance in cerebellum dependent eyeblink conditioning, with greater effects observed in global CB1 KO mice. These impairments were observed both in terms of the percentage and amplitude of CR's, but no

differences were detected in terms of the timing of these responses, when in comparision to the control groups.

Key Words: Eyeblink Conditioning; Cerebellum; Endocannabinoids; CB1R's; Plasticity

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ABBREVIATIONS LIST

CR: conditioned responses

2-AG: 2-arachidonoylglycerol

a6 CB1KO: mice that don't express CB1 receptors, specifically at the granule cells

AEA: arachidonoylethanolamide; anandamide

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

BLA: basolateral amygdala

Ca^{2+}: calcium

CB1: type 1 endocannabinoid receptor

CB1KO: mice that don' express CB1 receptors globally

CB2: type 2 endocannabinoid receptor

CR: conditioned response

CS: conditioned stimulus

Db: decibels

DAG: diacylglycerol

DCN: deep cerebellar nuclei

DSE: depolarization-induced suppression of excitation

DSI: depolarization-induced suppression of inhibition

EMG: electromyography

GABA: gamma-aminobutyric acid

IN: interpositus nucleus

IO: inferior olive

IPSC's: inhibitory post-synaptic currents

ITI: inter-trial interval

Khz: kilohertz

KO: knocked out

LED light: Light emitting diode light

LTD: long-term depression

LTD_i: long-term depression of inhibition

LTP: long-term potentiation

MF-DCN: synapse between Mossy fibers and the Deep cerebellar nuclei

mGluR: metabotropic glutamate receptor

NAPE: N-arachidonoyl phosphatidylethanolamine

NO: nitric oxide

N.S: not statistically significant

PF: Parallel fibers

PF-Pkj: synapse between Parallel fibers and the Purkinje cells

PIP2: phosphatidylinositol-4 5-bisphosphate

Pkj: Purkinje cell

SSE: synaptically evoked suppression of excitation

UR: unconditioned response

US: unconditioned stimulus

VOR: vestibule-ocular reflex

FIGURES AND TABLES INDEX

INTRODUCTION

In this introduction section I will start by introducing basic concepts of associative learning, and important aspects of the eyeblink conditioning behavior. I will then move on to explain the cerebellar circuit, and how it computes stimuli information during eyeblink conditioning. Finally, a chapter dedicated to endocannabinoids will elucidate its synthesis, and CB1receptors signaling, and how they modulate plasticity in the cerebellum and in delay eyeblink conditioning.

CLASSICAL EYEBLINK CONDITIONING

CLASSICAL CONDITIONING

Classical conditioning is a well-known form of associative learning, first described by Pavlov in the beginning of the 20th century (Kandel et al. 2000). In classical conditioning, an initially neutral stimulus – the conditioned stimulus (CS)- is successively paired with an unconditioned stimulus (US), until the repeated presentation of this pairing results in learning of a conditioned response (CR) (Kishimoto & Kano 2006; Kandel et al. 2000; Boele et al. 2010). Initially the CS produces no overt response - or a weak response, usually unrelated to the response that will be learned- (Kandel et al. 2000). On the other hand, the US, presented after the CS, results in a strong, consistent response, which is called the unconditioned response (UR) (Kandel et al. 2000). These unconditioned responses are innate and reflexive responses that are elicited without learning. On the other hand, the conditioned response (CR), is a learned behavior, that is developed in response to the previously neutral CS (Boele et al. 2010; Kandel et al. 2000).

CLASSICAL CONDITIONING OF THE EYELID

Eyeblink conditioning is a form of associative learning in which the subject develops a gradually stronger eyelid closure - the conditioned response (CR) - in response to a previously neutral CS, that can be either a tone or a light (Boele et al. 2010). The blink eliciting US is usually a mild electrical shock to the eyelid (Kishimoto & Kano 2006; Kishimoto et al. 2001; Koekkoek et al. 2003) or an air puff directed to the cornea of the eye (Chettih et al. 2011; Schonewille et al. 2011).

The CR is developed after several pairings of the CS and the US. Although during early trials there is only a blink in response to the US – the unconditioned response (UR)-, throughout sessions, a conditioned blink starts to develop in response to the CS (**Fig.1**).

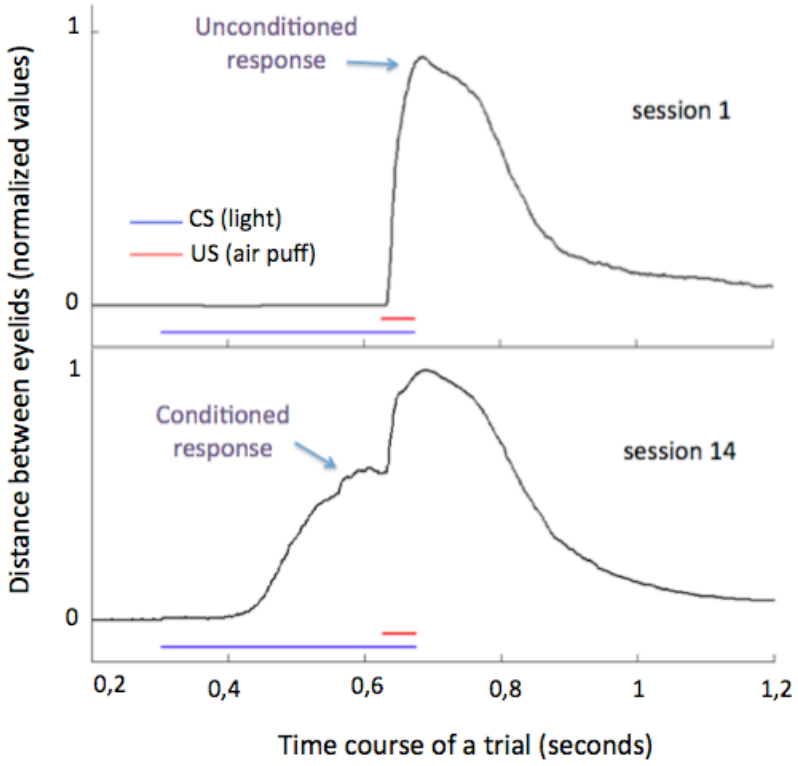

FIGURE 1.EYELID MOVEMENTS IN AN EYEBLINK CONDITIONING EXPERIMENT DURING INITIAL AND LATER STAGES:

The image represents the typical waveform of the eyelid movement during a trial in session 1 and at a later session. At session 1 only an Unconditioned Response is observed, while at a later session a Conditioned Response is also present. The y axis represents the normalized values of the distance between eyelids (0 is fully opened eye, 1 is maximum eyelid closure); The x axis represents the time elapsed form the onset of the trial in seconds.

Eyeblink conditioning can be either a trace or a delay paradigm. In the trace paradigm there is an interval of a few milliseconds between the end of the CS and the start of the US, while in the delay paradigm the CS and US co-terminate. While delay eyeblink conditioning is known to dependent on an intact cerebellum (McCormick et al. 1981; McCormick & Thompson

1984; Chen et al. 1996), the trace protocol also requires a well-functioning hippocampus (Boele et al. 2010). For the purpose of this thesis, *eyeblink conditioning* refers to the delay paradigm, unless stated otherwise.

Other extra-cerebellar structures may also play a role in modulating delay eyeblink conditioning, including the amygdala that seems to be particularly important to determine the effectiveness of the CS and to influence the state of arousal during training (Boele et al. 2010).

CELLULAR CIRCUIT OF THE CEREBELLUM

CIRCUITS INVOLVED IN EYEBLINK CONDITIONING

The cerebellar circuit comprises the cerebellar cortex and the deep cerebellar nuclei (Carey & Lisberger 2002).

Purkinje cells are the only output from the cerebellar cortex to other brain regions, receiving excitatory signals from two distinct classes of afferents - the mossy fibers and the climbing fibers – and then projecting to the deep cerebellar nuclei (DCN) through inhibitory connections (Purves et al. 2004) **(Fig2.)**.

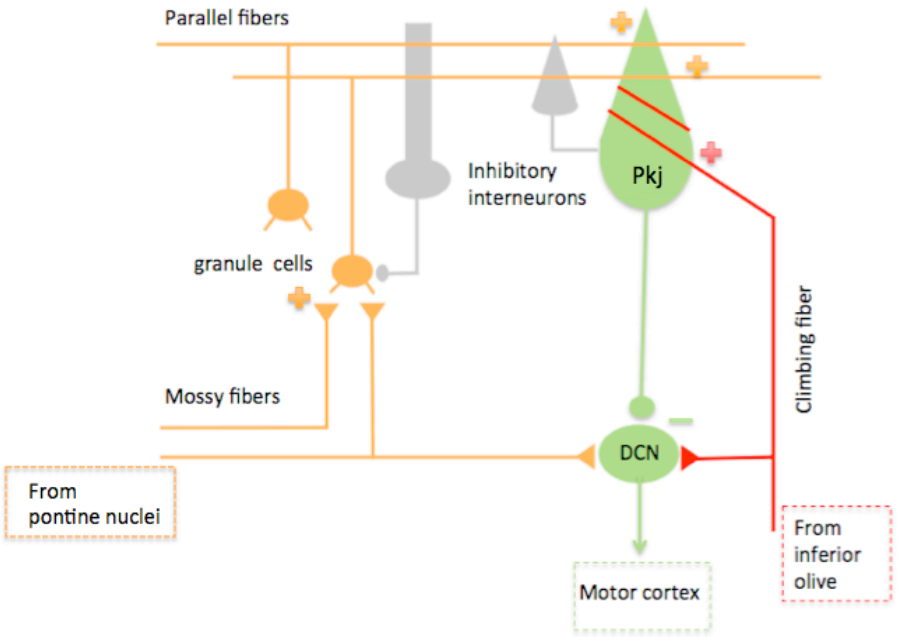

FIGURE 2.SCHEMATIC REPRESENTATION OF THE CEREBELLAR CIRCUIT:

Purkinje cells are the main output from the cerebellar cortex, and they receive excitatory input from two main pathways: The Mossy Fibers-Parallel fibers pathway , and Climbing Fibers. The deep cerebellar nucleus receives inhibitory projections from the Purkinje cells and also excitatory collaterals from both Mossy and Climbing fibers. The inhibitory interneurons in the cortex modulate Purkinje cells inputs and provide inhibitory feedback to the Parallel fibers. (+) excitatory synapse; (-) inhibitory synapse; Pkj cell: Purkinje cell; DCN: Deep cerebellar nuclei. *Adapted from Carey, M.R (2011).*

Climbing and mossy fibers are responsible for the inputs that arrive to the cerebellar cortex, converging important sensory information onto the Purkinje cells. Each of these pathways carries different information: mossy fibers-Parallel fibers pathways carries information concerning the CS, while Climbing fiber carry information about the US. In this way, information about the CS and US arrives to the cerebellum by means of the ponto-cerebellar and olivo-cerebellar circuits, respectively (Carey 2011; Raymond et al. 1996).

Mossy fibers arise both from the pontine nuclei in the brainstem - which in turn receive projections from the cerebral cortex – and from the spinal cord. The CS signals, specifically, are carried out from primary sensory cortical areas to the pontine nuclei. Projecting from the pontine nuclei, mossy fibers then enter the cerebellum through the middle peduncle. In the cerebellar cortex, mossy fibers synapse with granule cells, whose axons give rise to the Parallel fibers. Parallel fibers carry information to the Purkinje cells, connecting to numerous of these neurons. At the same time, a single Purkinje cell receives synapses from thousands of parallel fibers. Additionally, although to a lesser extent, mossy fibers' collaterals also project to the interposed nuclei (IN) of the DCN. Parallel fibers also project to inhibitory interneurons, which in turn contact with Purkinje cells and provide inhibitory feedback to granule cells (Carey 2011; Raymond et al. 1996; Purves et al. 2004; Boele et al. 2010).

Climbing fibers project from the inferior olive (IO), located in the brainstem. Information about the unconditioned air puff stimulus is received by the trigeminal nerve nucleus (TrN), and then carried through the dorsal accessory inferior olive (IO) to Climbing fibers. Climbing fibers then cross the inferior peduncle entering the cerebellum, and the information concerning the US is conveyed both directly to the Purkinje cells, or through climbing fibers collaterals to the deep cerebellar nuclei (DCN), specifically to the interposed nuclei (IN).

Unlike Parallel fibers, each Purkinje receives input from only one Climbing fiber, and at the same time, one single Climbing fiber reaches only a few Purkinje cells. A pathway going from the deep nucleus back to the inferior olive creates a recurrent olivo-cerebellar circuit (Carey 2011; Purves et al. 2004; Raymond et al. 1996; Carey & Lisberger 2002).

SITES OF PLASTICITY AND COMPONENTS OF THE BEHAVIOR

So far several synapses within the cerebellar circuit have been described to be plastic, and are thought to play a role in different components of motor learning (Hansel et al. 2001; Carey & Lisberger 2002). Out of the different forms and sites of plasticity, Long-term depression (LTD) at the Parallel fiber to Purkinje cell synapse has been hypothesized for many years to play a central role in cerebellar motor learning (Carey & Lisberger 2002; Carey 2011), although recent work seems to suggest otherwise (Schonewille et al. 2011).

According to the Marr-Albus-Ito theoretical model for cerebellar learning, conjunctive activation of parallel fiber and climbing fiber inputs leads to long-term depression (LTD) of the parallel fiber to Purkinje cell synapse (Carey 2011; Raymond et al. 1996).
Ito and his collaborators first described Cerebellar LTD. They observed that coincident low frequency stimulation to climbing and parallel fibers resulted in a decrease in the strength of the Parallel fiber/Purkinje cell synapse (Ito et al. 1982). The overall outcome for the circuit is an increase in DCN firing, presumably caused by the decrease of inhibitory Purkinje cell inputs (Hansel et al. 2001). In terms of behavioral output, Climbing fibers signal error and their simultaneous activation with parallel fibers would contribute to the decrease of the strength of parallel fibers inputs that were consistently associated with errors. Decreasing the strength of these inputs from Parallel fibers to Purkinje cells would alleviate the inhibitory input from Pkj cells to the DCN and consequently send a motor command to cortical areas of

the brain, leading to the generation of a new learned motor response (Carey 2011; Carey & Lisberger 2002).

Initially this form of LTD at the Parallel fiber to Purkinje cell synapse was thought to be the only modifiable synapse, and a lot of research was made focusing on the assumption that it was the sole neural mechanism underlying learning (Carey & Lisberger 2002).
Despite this, in vitro studies have shown that there are other sites where long-term plasticity takes place - both in cerebellar cortex and at the deep nuclei - (**Fig.3**) (Hansel et al. 2001). On the other hand, there is also an increasing body of evidence suggesting that particular features of eyeblink conditioning seem been linked to different sites of plasticity within the cerebellar circuit (Carey & Lisberger 2002).

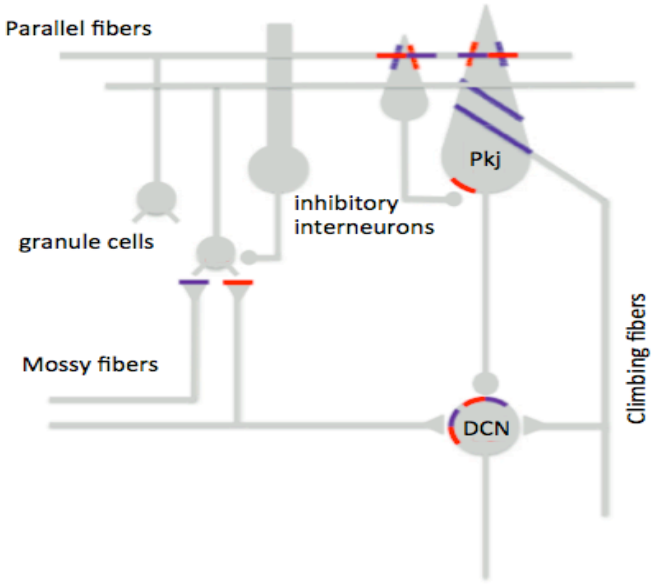

FIGURE 3.SITES OF THE CEREBELLAR CIRCUIT WHERE LONG-TERM PLASTICITY HAS BEEN DESCRIBED TO OCCUR:

The figure is a schematic representation of the places where long-term plasticity has been described within the cerebellar circuit. Long-term plasticity has been described to occur in all synapses onto Purkinje cells (Mossy fibers, Climbing Fibers and interneurons). It has also been described in the synapses received by the Deep Cerebellar Nuclei (specifically from Mossy fibers and the Purkinje cells), between Parallel fibers and the interneurons, and the connections between Mossy fibers and Granule cells. Blue: Long-term Depression, Red: Long-term Potentiation. DCN: Deep cerebellar nucleus, Pkj: Purkinje cells. *Reproduced from Carey, M.R (2011)*

Timing, for instance, is a learned feature of eyeblink conditioning, where the blinks have to be timed accurately to match a specific interval between the onset of the CS and the onset of the US (Koekkoek et al. 2003). This specific component of the CR can be dissociated from its expression (or amplitude), and the two processes seem to be encoded at different sites in the cerebellar circuit. Mauk and colleagues proposed the existence of two sites of learning sites - the cerebellar cortex and the interposed nuclei - each responsible for two different features of eyeblink conditioning: timing and amplitude of the conditioned responses (Medina & Mauk 2000). According to the model, timing of the CR depends on plasticity mechanisms that modulate Pkj cells output in a temporal manner, which disinhibits the DCN only at the appropriate times. The expression of CR, on the other hand would depend on mossy fiber to DCN strengthened connections (Carey & Lisberger 2002). There is also some experimental evidence supporting this hypothesis (Bao et al. 2002).

Amplitude is also a component of the CR that can be regarded as the response gain since the CR is not an "all-or-non" response, and its amplitude increases monotonically from minimum to maximum eyelid closure (Kreider & Mauk 2010). In recent work by (Kreider & Mauk 2010), the authors developed a training procedure where the delivery of the US is made contingent on the amplitude of the CR. The results showed that mice learn to condition their eyelid responses to match the targeted amplitudes. Because animals could also learn the amplitude of the responses when the CS was substituted by direct mossy fiber stimulation, and muscimol injections to the interpositus nucleus abolished previously acquired responses, the authors concluded that amplitude of the CR is a feature coded and stored by the cerebellum.

Extinction, on the other hand, is thought to be encoded at the olivo-cerebellar loop, through the recurrent pathway from the olivary nucleus to the cerebellar cortex, and from inhibitory interneurons in the deep cerebellar nuclei back to the inferior olive (Medina et al. 2002). Medina and associates showed that intra-olivary infusions with a GABA antagonist prevented extinction, while infusions in the same area using AMPA antagonist resulted in gradual loss of previously acquired CR's. Together with previous computational findings that both acquisition and extinction processes require a deviation of climbing fiber basal/background activity, the authors propose that an increased inhibitory input from the DCN to the Inferior Olive (IO) would be the teaching signal for extinction.

ENDOCANNABINOIDS AND PLASTICITY AT THE CEREBELLUM

ENDOCANNABINOID SYNTHESIS AND RELEASE

Endocannabinoids act as neuromodulators throughout the brain. They are retrograde neurotransmitters, released from postsynaptic neurons and acting at presynaptic terminals. **(Fig.4)** Endocannabinoids are lipid molecules derived from membrane phospholipids. In the central nervous system they can be either arachidonoylethanolamids (AEA's) – also known as anandamides - or 2-arachidonoylglycerol (2-AG). These two types of endocannabinoids have different pathways of synthesis. Phosphatidylethanolamine, the initial precursor of AEA, is converted to N-arachidonoyl phosphatidyethanolamine (NAPE) by the enzyme N-Acyltransferase in a Ca^{2+} dependent manner, and the subsequent cleavage of NAPE originates AEA. 2-AG endocannabinoids are generated by the sequential hydrolysis of Phosphatidylinositol-4,5-bisphospshate (PIP2) and diacylglycerol (DAG).

Endocannabinoids release can be triggered by two different protocols: either by an increase of intracellular Ca^{2+} levels as a consequence of the neuron depolarization, or by activation of Gq-coupled receptors, such as group I metabotropic glutamate receptors (mGluR1) **(Fig.4).**

(El Manira & Kyriakatos 2010)

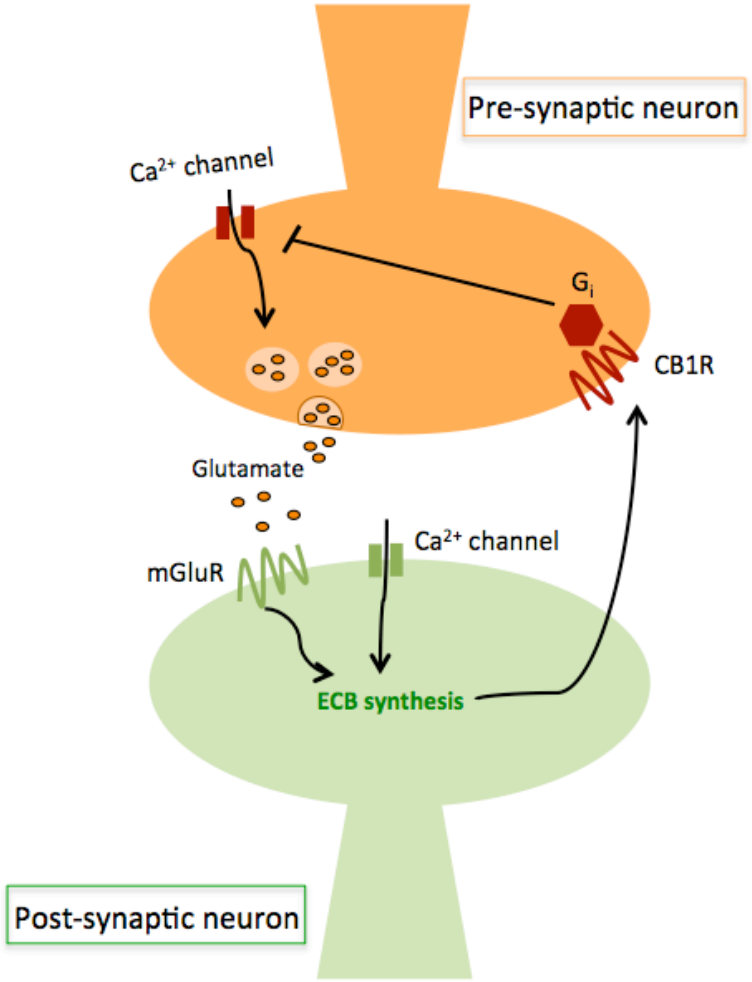

FIGURE 4.CB1 RECEPTOR SIGNALING:

Endocannabinoids are retrograde messengers: they are released from post-synaptic neurons and act on receptors located at pre-synaptic terminals. Endocannabinoid synthesis and release can be triggered either by an increase in intracellular Ca^{2+}, or by activation of metabotropic glutamate receptors (mGluR). Upon their release, endocannabinoids can act on type 1 cannabinoid receptors (CB1R) which are usually coupled to G-inhibitory proteins (G_i). Activation of G-inhibitory proteins inhibits neurotransmitter release from the pre-synaptic terminals, by decreasing the influx of $Ca^{2'}$ to the pre-synaptic neuron.

ENDOCANNABINOID RECEPTORS

To date, two endocannabinoid receptors are known: CB1 and CB2 receptors. CB1 receptors are expressed virtually throughout the central nervous system, acting predominately at presynaptic terminals. Although they are primarily coupled to Gi/o proteins, they can also be coupled to Gq proteins to induce Ca^{2+} release from intracellular stores (El Manira & Kyriakatos 2010). CB2 receptors expression is constricted to more specific regions, such as the brainstem, and the levels of expression are much lower than the ones observed for CB1 receptors. Like CB1 receptors, they are also primarily coupled to Gi/o, but their function in the central nervous system is still not well defined. Although cannabinoid signaling in the brain is mainly mediated by CB1 receptors, additional endocannabinoid receptors may also be present.

(El Manira & Kyriakatos 2010)

ENDOCANNABINOID SIGNALING AND PLASTICITY

Endocannabinoids are known to play an important role in synaptic plasticity. They were first implicated in a form of short -term plasticity, observed both in hippocampus and in the cerebellum. This form of plasticity is called depolarization-induced suppression of inhibition (DSI) and consists in a suppression of inhibitory GABAergic transmission onto Purkinje cells and hippocampal CA1 pyramidal cells, that lasts about 10 seconds. DSI is induced by an increase of Ca^{2+} levels at the postsynaptic neurons – in the cerebellum, these are the Purkinje cells - that leads to the release of retrograde messengers. These retrograde messengers are endocannabinoids and they act by activating presynaptic CB1R's, that are located at the axons of GABAergic interneurons. Upon their activation by retrograde signaling, these receptors suppress spontaneous IPSC's, which arise primarily from basket cells and stellate cells in the molecular layer of the cerebellum.

(Diana et al. 2002; a C. Kreitzer & Regehr 2001; Yoshida et al. 2002)

A similar form of short-term plasticity, depolarization-induced suppression of excitation (DSE), is also dependent upon endocannabinoids retrograde signaling. DSE is also described in cerebellar Purkinje cells. The result is the inhibition of climbing fibers and parallel fibers EPSC's, for a time course similar to DSI.
(A. C. Kreitzer & Regehr 2001; Maejima et al. 2001)

Suppression of excitation can also be induced by activation of Gq-coupled receptors, in a process called synaptically evoked suppression of excitation (SSE) (Carey et al. 2011; Brown et al. 2003). In this process, synaptic activation, through high frequency stimulation of parallel fibers, was shown to result in the activation of mGluR1 in the Purkinje cells, leading to the synthesis of endocannabinoids, presumably 2-AG. Released endocannabinoids then act at presynaptic CB1 receptors, inhibiting neurotransmitter release at parallel fibers.

Besides short-term plasticity, endocannabinoids also play a role in long-term forms of plasticity. Specifically, associative parallel fibers and climbing fibers excitatory inputs to Purkinje cells, result in endocannabinoids-mediated long-term depression (LTD) at the Purkinje cells. LTD at Parallel fiber to Purkinje cells synapse shares some common features with the transient associative form of retrograde inhibition, SSE (Safo & Regehr 2005; Brenowitz & Regehr 2005). Just like parallel fibers SSE, LTD at parallel fiber to purkinje cell synapse is mGluR1 dependent and requires the rising of Ca^{2+} intracellular levels in the purkinje cells (Safo & Regehr 2005; Aiba et al. 1994; Ito 2001). They also share an important associative property, as they are best induced when Parallel fibers are activated within several milliseconds of climbing fiber stimulation (Ito 2001; Wang et al. 2000; Brenowitz & Regehr 2005). Furthermore, LTD is blocked by the use of a CB1R antagonist, by inhibiting the

synthesis of 2-AG endocannabinoids and is absent in mice lacking CB1R's, indicating that both in SSE and in LTD at the parallel fibers to purkinje cells synapse, 2-AG is released from Purkinje cells to act at CB1R's (Safo & Regehr 2005) .

Despite the similarities between the two processes, SSE is expressed presynaptically at the parallel fibers and LTD is thought to be expressed postsynaptically (Ito 2001). Adding to this, CB1R's are expressed in Parallel fibers boutons but have not yet been detected in Purkinje cells (Safo & Regehr 2005). Because so far CB1R's have only been detected at Parallel fibers, but LTD is expressed postsynaptically at Purkinje cells, it is possible that a second messenger is also involved in this process, acting as an anterograde messenger. NO is a good candidate, as it is thought to be released from Parallel fibers upon activation of CB1R, acting in Purkinje cells (Safo & Regehr 2005; El Manira & Kyriakatos 2010). Supporting this idea, cerebellar LTD is blocked by disruption of NO signaling (Daniel et al. 1998). Parallel fibers release glutamate upon stimulation which acts upon mGluR1 and AMPA receptors located at the Purkinje cells. mGluR1activation then leads to endocannabinoid release which act retrogradely at CB1R'a located at the Parallel fibers, and NO is thought to be released from these cells to act on Purkinje cells. At the same time, Climbing fibers activation leads to Ca^{2+} influx through voltage gated channels. LTD is expressed postsinaptically at Purkinje cells, as a consequence of the internalization of AMPA receptors by clathrin-mediated endocytosis (Hansel et al. 2001).

More recent electrophysiological experiments, using mice where CB1R's were selectively eliminated from granule cells, showed that both short and long term-plasticity at synapses between Purkinje cells and Parallel fibers were impaired (Carey et al. 2011). This suggests that in fact CB1R's that are involved in LTD are located presynaptically, at the parallel fibers.

In summary, CB1R's are involved and are necessary for important plasticity mechanisms taking place in the cerebellar cortex. Endocannabinoids retrograde signaling can influence the amount of transmitter release, producing local changes in synaptic strength. These changes, that can be expressed either pre or postsynaptically, are thought to account for several aspects of motor learning (as previously mentioned here).

THE ROLE OF ENDOCANNABINOIDS IN EYEBLINK CONDITIONING

In the large field of neuroscience, neuronal plasticity has been long believed to be the cellular and molecular basis for learning. Synaptic plasticity taking place at the cerebellar cortex can be observed at different synapses onto the Purkinje cells – the sole output from the cerebellar cortex-, and at different timescales – long versus short-term plasticity-. These forms of plasticity are thought to account for several aspects of cerebellum-dependent motor learning in behaviors such as delay eyeblink conditioning or the vestibulo-ocular reflex (VOR)(Raymond et al. 1996).

As it was described above, endocannabinoids have been proven to play a role in different forms of plasticity at the cerebellar cortex (Carey et al. 2011). If it is in fact true that this forms of plasticity are underlying motor learning, one would expect to see direct changes in behavioral tasks, once these neuromodulators signaling is affected.

Delay eyeblink conditioning is a particular type of behavior that relies upon an intact cerebellum. Associative learning is necessary for the expression of this behavior, and several sites of plasticity within the cerebellum have been identified, that seem to be mediating different features of eyeblink conditioning (Carey & Lisberger 2002). In fact, in 2006 Yasushi Kishimoto and Masanobu Kano published their work, describing how delay eyeblink conditioning was impaired in mice that lacked CB1R's (Kishimoto & Kano 2006). They assessed both CB1R global KO mice and mice injected with the CB1 antagonist in delay and

trace eyeblink conditioning. Both groups showed impaired acquisition in delay eyeblink conditioning, but not in the hippocampus-dependent protocol of trace eyeblink conditioning. Furthermore, in mice injected with the CB1 antagonist SR141716A, it was possible to conclude that despite the impairments in acquisition, extinction had not been influenced. The fact that impaired performance is observed in cerebellum-dependent delay eyelid conditioning, but CB1R KO mice present normal learning in trace eyelid conditioning, suggests that the CB1R's required for this behavior are located within the cerebellum. With these results, the authors conclude that endocannabinoid signaling through CB1R's is essential for cerebellum-dependent discrete motor learning, especially for acquisition (Kishimoto & Kano 2006). Moreover Kishimoto and Kano's work contributed to establishing a direct link between previous electrophysiology findings, where endocannabinoids signaling was observed to mediate long and short-term forms of plasticity within the cerebellum, and concrete behavior evidence that CB1R's are modulating learning in the cerebellum. The question then arises as to how, and where in the cerebellum - in which cell types- are these receptors regulating motor learning.

OBJECTIVES

Here we aim to understand how CB1R's are modulating learning in cerebellum-dependent delay eyeblink conditioning – which components of the behavior are being affected and in what way -, and in which specific cell types are these receptors exerting these effects.

To answer the above-mentioned questions, delay eyeblink conditioning was assessed in transgenic mice lacking CB1R's. Percentage, amplitude and timing of the CR's was analyzed in global and granule cell specific CB1 KO mice and the results were compared to those of the respective controls.

Specific Aim 1:

Assess eyeblink conditioning in Global CB1 KO by looking at the percentage of Conditioned responses (%CR) obtained, replicating previously published results (Kishimoto & Kano 2006), under the present experimental conditions.

Specific Aim 2:

Extend the previously published experiments, by assessing the amplitude and timing components of the CR in Global CB1 KO mice.

Specific Aim 3:

Assess Granule cell specific CB1 KO mice (Alpha6 Cre;CB1 fx) in eyeblink conditioning, measuring the percentage (%CR), amplitude and timing of the CR's.

METHODS

ANIMALS

For the present experiments, C57BL/6 mice of two strains were used: global CB1 KO mice (n=14), and Alpha6 cre;CB1 fx mice (n=17). Mice ages in the first acquisition sessions were between 68 days (minimum) and 139 days (maximum). Animals were individually housed in cages with food and water *ad libitum*, under a 12 hours light/dark inverted light cycle (lights on at 8:00 P.M.).

Data from males and females was plotted and grouped together, as no meaningful differences were observed between genders.

GLOBAL KO

Global CB1R KO mice are transgenic mice that lack CB1R's all over the brain. CB1 -/- mice (CB1KO) and their controls, CB1 +/+, were obtained by intercrossing heterozygous breeding pairs (CB1 +/-) that were previously purchased by the laboratory.

CONDITIONAL KO

Alpha6 cre;CB1 fx mice are cell-type specific CB1R KO transgenic mice, in which cerebellar granule cells are specifically targeted. Alpha6 cre;CB1fx mice were generated using the Cre/loxP recombination system (**Fig.5**). Some of the resulting progeny will lack CB1R's receptos specifically at granule cells (a6 CB1KO). The controls were mice from the same strain, but that did't lack CB1R's.

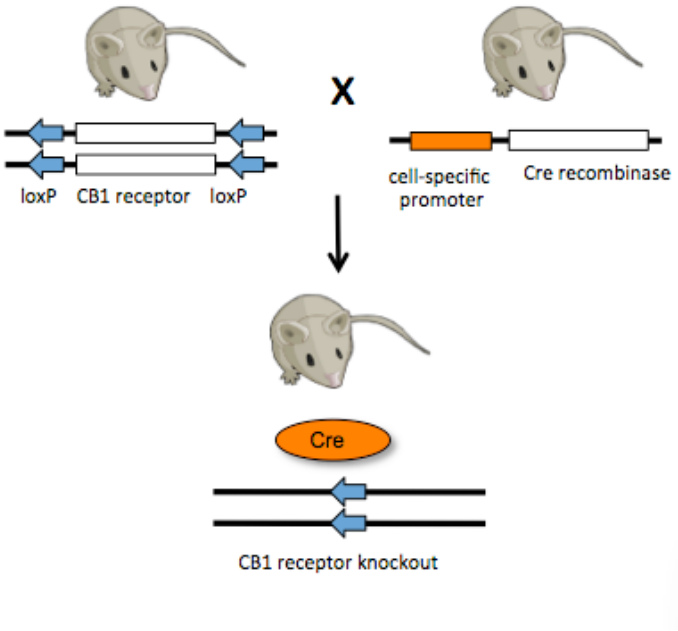

FIGURE 5.CRE/LOXP RECOMBINATION SYSTEM:

The Alpha6 Cre CB1;fx strain was generated using the Cre/loxP recombination system. In this genetic approach, mice carrying loxP sequences that flank the gene coding for CB1R's, were crossed with mice where Cre Recombinse expression was restricted to a specific cell type. The gene coding for the Cre Recombinase enzyme was in a different chromossome. As a result some of the progeny will have both of CB1 alleles flanked by loxP sequences, and also express Cre Recombinase. In the cells expressing Cre Recombinase , this enzyme removes the flanked CB1 gene .

HEAD FIX IMPLANT SURGERY

In order to head fix the animals a rectangular custom-cut metal plate was transversally attached to the skull, using dental cement.

Mice were anesthetized for surgery using isoflurane, and surgery proceeded with animals placed on a surgery station with continuous flow of oxygen and isoflurane. Eyes were abundantly covered with FRAKIDEX in the beginning of the surgeries to prevent them from drying.

A small circle of skin was cut off from the top of the head to expose the skull.

In order to increase the surface of adhesion a dental drill was used to mildly scratch the bone. After this a thin layer of glue was placed on top of the skull, followed by a layer of dental cement. Then the metal plate was placed, and an extra layer of cement was added on the top.

A painkiller – DOLOREX -was injected intraperiotoneally approximately 30min before the end of the surgery.

Animals only started habituation and handling at least 24 hours after the surgery and only if they were fully recovered.

SETUP

Behavioral assessment of the eyeblink conditioning was performed in a sound proof box with a surveillance camera. Mice were head-fixed to a custom-built treadmill so that they could walk during the sessions, as it seemed to make them less agitated.

A blue LED, positioned towards the right eye, was used as the CS, instead of an auditory tone, as it avoids the presence of an auditory startle reflex (Boele et al. 2010). For the US, a 50ms, 50psi air puff was chosen. The air puff was delivered using a Picospritzer, through a needle that was also positioned to the center of the right eye.

Videography was used as the recording method for the present experiments: eyelid movements were recorded using a high-speed camera (900 frames per second). This recording method provides reliable data concerning not only the percentage, but also amplitude and timing information about the conditioned responses. In **Fig.6** we can see a labeled photo of one of the experimental setups.

Labview was used to trigger and control all of the hardware in a synchronized way.

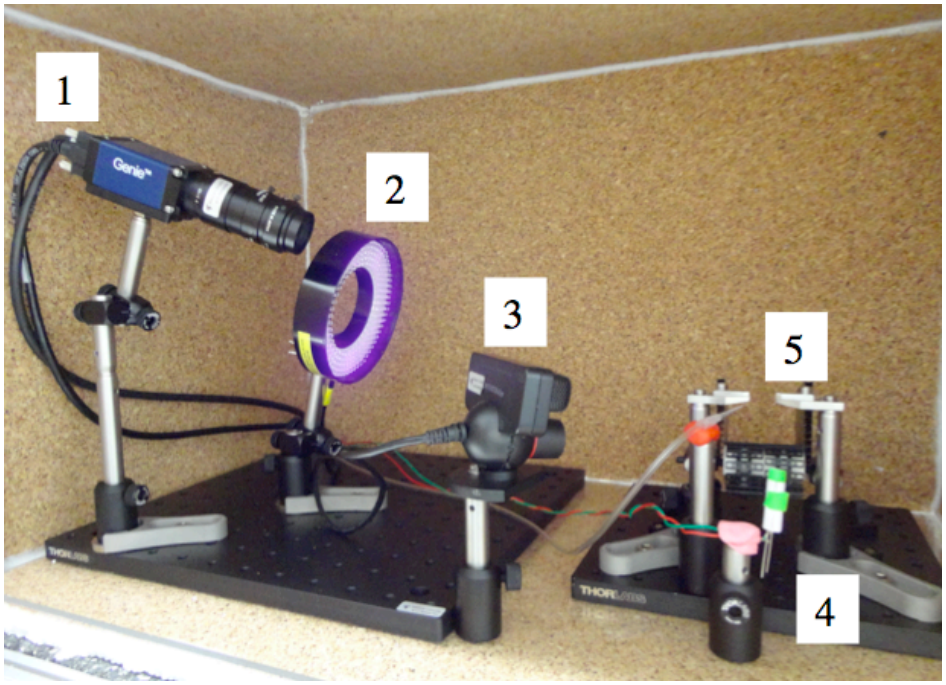

FIGURE 6.EXPERIMENTAL SETUP:

1) High speed camera; 2) Infrared lights; 3); Surveillance camera; 4) Treadmill, LED and air puff delivery system; 5) Head fix apparatus

CONDITIONS

Delay eyeblink conditioning was assessed for Alpha 6 cre;CB1 fx and global CB1R KO mice, and its respective controls. Conditioning experiments consisted of 4 different phases: handling and habituation sessions, acquisition/training sessions, test sessions, and extinction sessions (**Table.1**).

Phase	Number of sessions	%CS only trials
Habituation sessions	4-5 daily sessions	-
Acquisition sessions	20 sessions	10%
Test sessions	2 sessions	50%
Extinction sessions	3 sessions	70%, 90%, 100%

TABLE 1.DIFFERENT PHASES OF THE CONDITIONING EXPERIMENTS AND PERCENTAGE OF UNPAIRED CS - ONLY TRIALS

HABITUATION SESSIONS

Before starting any behavioral experiments, mice were first handled for 15-20min.

Habituation sessions were then performed, consisting of 4 to 5 daily sessions in which mice were head fixed in the behavior box for 10,15,20 and finally 30min. No light nor air puff were presented during these sessions.

In order to facilitate head fixing and to avoid stressing out the animals, head fixing was done under light isoflurane anesthesia.

ACQUISITION/ TRAINING

During acquisition session animals learn the association between the CS and the US and develop the CR, after several pairings of these two stimuli.

Each session comprised 110 trials divided in blocks of ten trials each, with 9 CS-US pairings and 1 CS only trial. The CS only trials in each block were presented in a randomized way. Unpaired CS-only trials make it possible to observe CR's more clearly, as they are not contaminated by the presence of reflexive unconditioned response (UR) to the US.

Each trial was separated from the previous one by an inter-trial interval (ITI) of [5-10] seconds (randomized values).

Mice were lightly anesthetized using isoflurane, to facilitate head fixing and to avoid causing any additional stress to them. Importantly, mice were left head fixed in the behavior box for 15min before the actual sessions started (this assured that mice were fully awake and also not agitated, by the time the session started).

Once mice were head fixed, and before the beginning of the session, the direction of the air puff was adjusted individually for each mouse to make sure that the US was eliciting a proper eye blink.

TEST SESSIONS

Test sessions consisted of 2 sessions with 50% unpaired CS-only trials, which were presented in a randomized manner, and with the same ISI and ITI as Acquisition/Training sessions. The purpose of these test sessions was to have an increased number of trials where no US iwas presented, and hence there was no UR's contaminating the CR.

EXTINCTION

Extinction sessions consisted of 3 sessions, identical to the previous acquisition and test sessions, but in which the percentage of unpaired CS-only stimuli was gradually increased throughout sessions. The first sessions was ran with 70% CS-only trials, followed by a session with 90%, and another one with 100% CS-only trials. By gradually increasing the percentage of unpaired CS-only trials, we hoped to slow down the extinction process, to facilitate the detection of possible CB1R's effects.

DATA COLLECTION AND ANALYSIS

Eyelid movements were recorded for each trial, using videography. To analyze the eyeblinks, the distance between the two eyelids was measured in pixels during each trial, using image analysis with MatLab. The recorded values for the distance between the two eyelids where normalized on a scale from 0 to 1, with 1 being the maximum eyelid closure.

To identify the CR's in each session the minimum distance between the two eyelids was defined as ≥ 0,1 (normalized pixel values: 0 is fully opened eyelid, 1 is maximum eyelid closure). Additionally, in order to be considered a CR, the eyelid closure had to occurr after the start of the CS and before the start of the US.

Averaged amplitude of the CR (avg amp) was calculated for each session as the mean value of the maximum eyelid closures occurring every trial. Only eyelid closures ≥ 0,1 (normalized pixel values), occurring after the start of the CS and before the onset of the US, were considered.

Timing information was extracted from CS-only trials of the 2 test sessions. It represents the averaged values of amplitude of eyelid closure obtained through the time course of a trial (time in milliseconds). Once again, only eyelid closures that reached an amplitude ≥ 0,1 (normalized pixel values) were considered.

Statistical analysis was performed to determine the significance of differences found between the KO and the control groups. T-Test analysis was performed in groups of 4 sequential acquisition sessions, 2 test sessions and 3 extinction sessions, because of the low number of subjects present in individual sessions. Differences were considered statistically significant when the calculated p-value >0,05.

RESULTS

The results are presented here in 2 groups:

- Assessing Global CB1 KO mice (CB1KO *versus* control mice)
- Assessing Alpha 6 cre;CB1 fx mice (a6 CB1KO *versus* control mice)

For each of these groups, results concerning percentage, amplitude and timing of the CR will be presented.

Data from both percentage and amplitude of the CR's is presented in 2 figures: one depicts the performance throughout the individual sessions, and another one depicts the averages of grouped sessions (groups of 4 sequential acquisition sessions, 2 test sessions, and 3 extinction sessions) with the respective results of statistical significance.

For the amplitude, data is only presented from session 5 onwards. This is because only trials where a CR was present were considered for the analysis of amplitude, and for the initial 4 sessions only a small percentage of CR's was expressed.

Results concerning analysis of timing are also presented in 2 figures: the first one representing the averaged waveforms of the eyelid movement from trials of test sessions where CR's are expressed; the second one representing the averaged latency times to peak amplitude of the CR's in test sessions (in seconds), and the statistical significance between the groups.

In order to replicate the previously published results by (Kishimoto & Kano 2006), initially a total of 14 mice from the Global CB1 KO strain were tested in eyeblink conditioning (n=4 controls; n=10 CB1KO). As mentioned before, experiments consisted of 20 acquisition sessions, followed by 2 test and 3 extinction sessions. One mouse from the CB1KO group ran only16 acquisition sessions, and no test or extinction sessions

Data concerning percentage, amplitude and timing of the CR's was analyzed and compared to that of controls.

Percentage of Conditioned Responses

Data concerning the averaged percentage of CR's per session (%CR) shows a decreased expression of CR's in Global CB1 KO mice (CB1KO), that fail to reach the same levels of performance as the control group (t-test p-values for grouped sessions, S1-S4:0,08; S4-S8:0,02; S9-S12:0,09; S13-S16:0,04; S17-S20:0,01) (**Fig.7** and **Fig.8).** No statistically significant differences were detected between Global CB1 KO mice (CB1KO) and the respective controls, at test or extinction sessions (t-test p-values for grouped sessions, T1-T2:0,14; E1-E3:0,13) (**Fig.7** and **Fig.8).**

Averaged amplitude of Conditioned Responses

Statistically significant differences were also detected between Global CB1 KO mice (CB1KO) and controls in data from averaged amplitudes of the CR's (avg amp), in both acquisition and test sessions (t-test p-values for grouped sessions, S5-S8:0,00; S9-S12:0,00; S13-S16:0,00; S17-S20:0,00; T1-T2:0,00) (**Fig.9** and **Fig.10**). During extinction sessions no differences were observed between Global CB1KO mice (CB1KO) and the control group (t-test p-values for grouped sessions, E1-E3:0,91) (**Fig.9** and **Fig.10)** .

Averaged timing of the CR's

Despite, as mentioned before, the averaged maximum amplitude of the CR's being higher in controls (**Fig.9, Fig.10** and **Fig.11**), no differences were found in latency to peak amplitude between the 2 groups (t-test p-value: 0,36) (**Fig.11** and **Fig.12**). The onset and offset time of the CR are also very similar in global CB1 KO and controls (**Fig.11**).

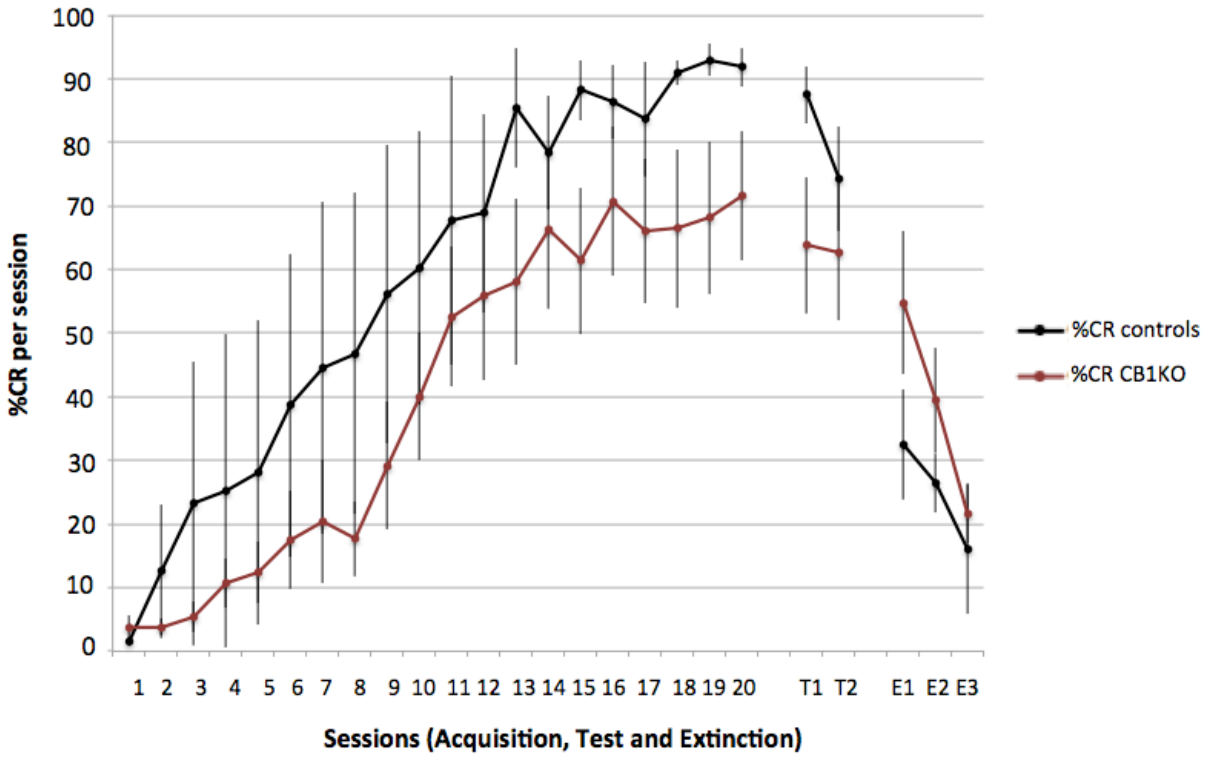

FIGURE 7.GLOBAL CB1 KO MICE - AVERAGED PERCENTAGE OF CONDITIONED RESPONSES (%CR) IN EACH SESSION:

Comparison of the percentage of CR's (%CR) in Global CB1 KO and control mice, throughout sessions. The y axis represents the averaged percentage of the CR's per session; The x axis represents session number (T:Test, E:Extinction). Red line, averaged percentage of CRs in Global CB1 KO mice (CB1KO); Black line, averaged percentage of CRs in control mice

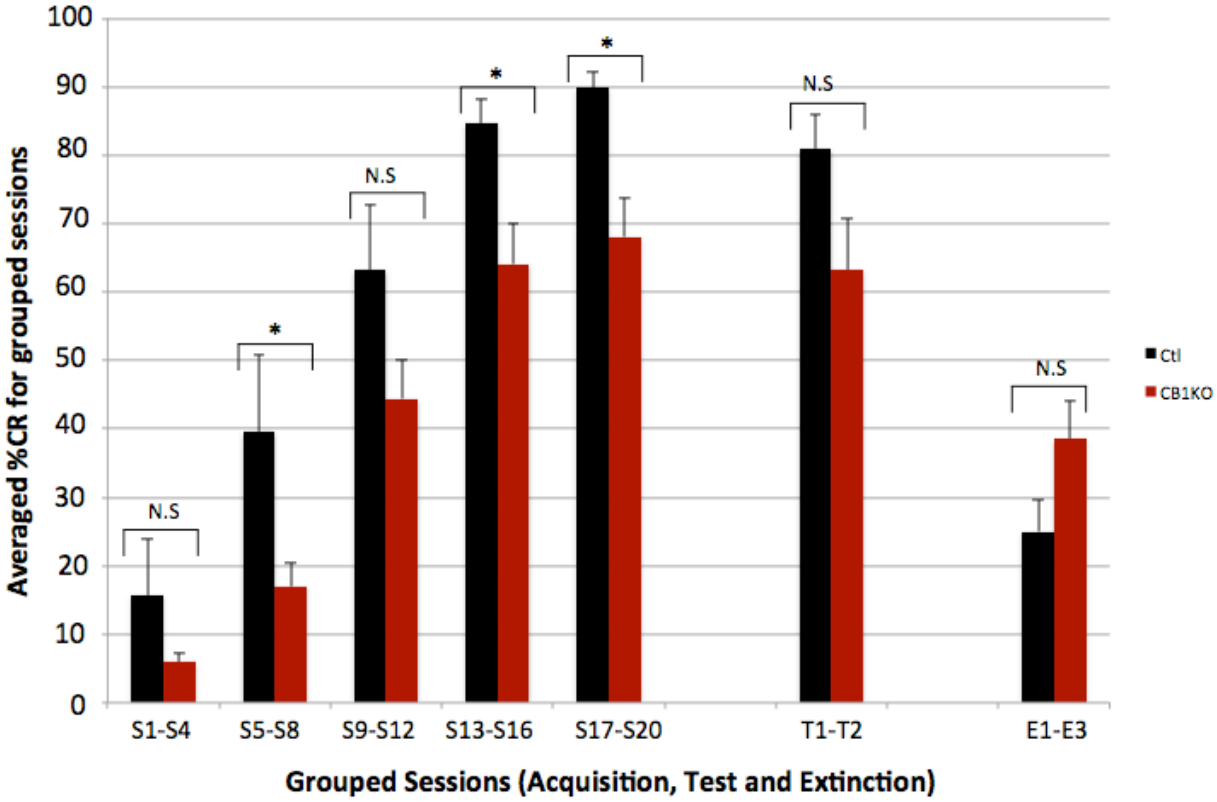

FIGURE 8.GLOBAL CB1 KO MICE - AVERAGED PERCENTAGE OF CONDITIONED RESPONSES (%CR) FOR GROUPED SESSIONS:

Comparison of percentage of CR's (%CR) in Global CB1 KO and control mice in groups of 4 acquisition sessions, 2 test sessions and 3 extinction sessions. The y axis represents the averaged percentage of the CR's on each group of sessions ; The x axis represents session number (S:Acquisition, T:Test, E:Extinction). Red bars, averaged percentage of CRs in Global CB1 KO mice (CB1KO) for the considered interval of sessions; Black bars, averaged percentage of CRs in control mice (Ctl) for the considered interval of sessions. N.S: differences are not statisitcally significant, p-value ≥0,05; (*): statistically significant differences, p-value <0,05

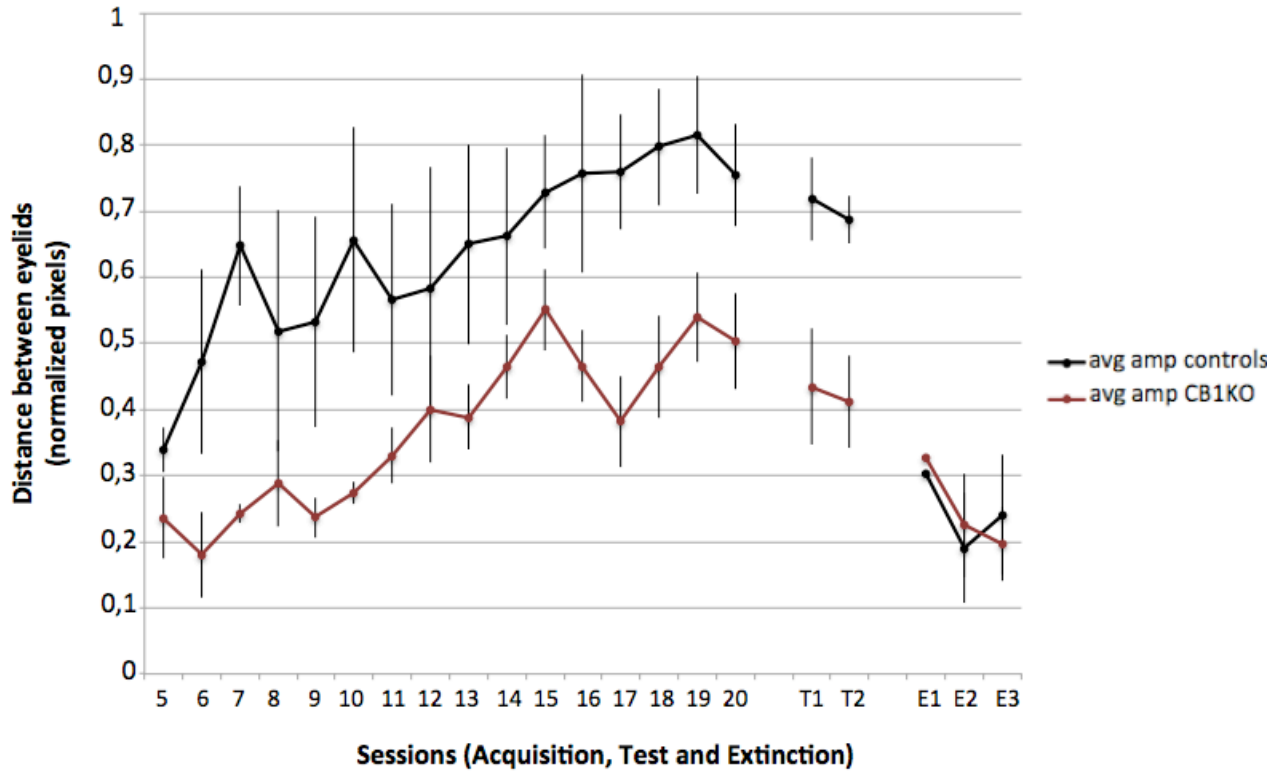

FIGURE 9.GLOBAL CB1 KO MICE - AVERAGED AMPLITUDE OF CONDITIONED RESPONSES (AVG AMP) IN EACH SESSION:

Comparison of the amplitude of the CR's (avg amp) in Global CB1 KO and control mice, from session 5 onwards. The y axis represents the normalized values of the eyelids distance in pixels (0 is fully opened eye, 1 is maximum eyelid closure) for each session; The x axis represents session number (T:Test, E:Extinction). Red line, averaged amplitudes of CR's per session, in Global CB1 KO mice (CB1KO) . Black line, averaged amplitudes of CR's per session, in control mice

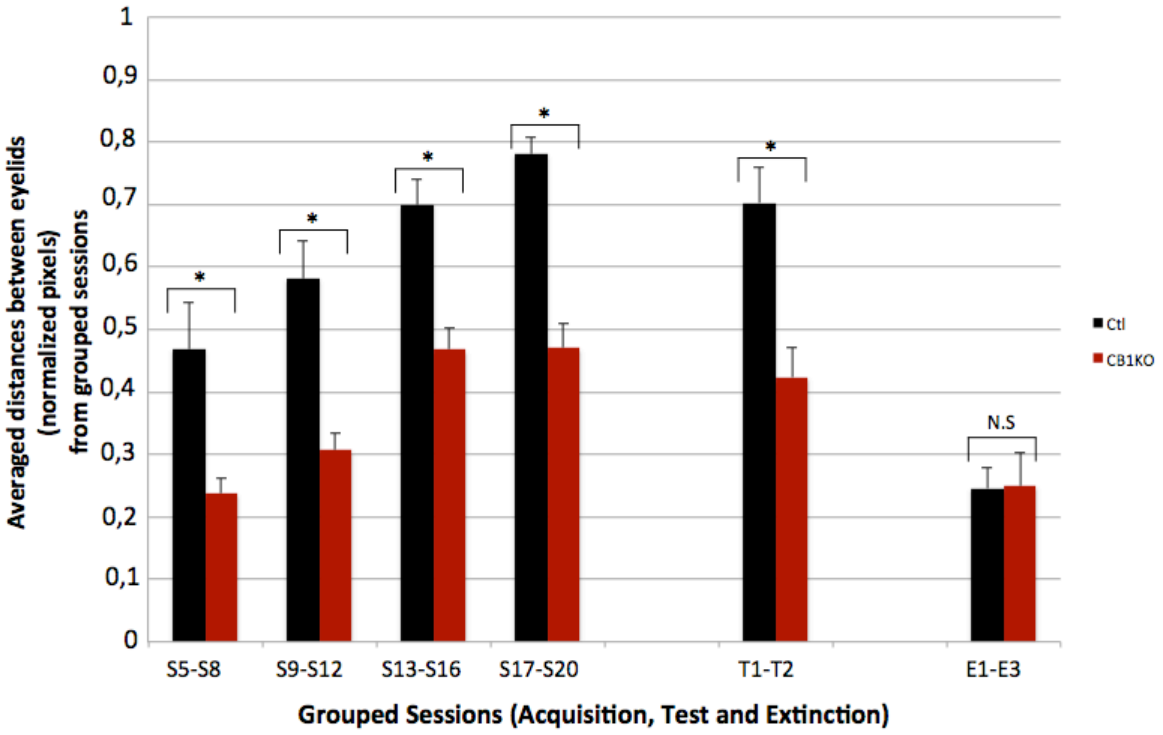

FIGURE 10.GLOBAL CB1 KO MICE - AVERAGED AMPLITUDE OF CONDITIONED RESPONSES FOR GROUPED SESSIONS:

Comparision of the amplitude of CR's in Global CB1 KO and control mice in groups of 4 acquisition sessions, 2 test sessions and 3 extinction sessions . The y axis represents the averaged values of the normalized distances between eyelids, for each group of sessions ; The x axis represents session number (S:Acquisition, T:Test, E:Extinction). Red bars, averaged amplitude of CRs in Global CB1 KO mice (CB1KO) for the considered interval of sessions; Black bars, averaged amplitude of CRs in control mice (Ctl) for the considered interval of sessions. N.S: differences are not statisitcally significant, p-value ≥0,05; (*): statistically significant differences, p-value <0,05

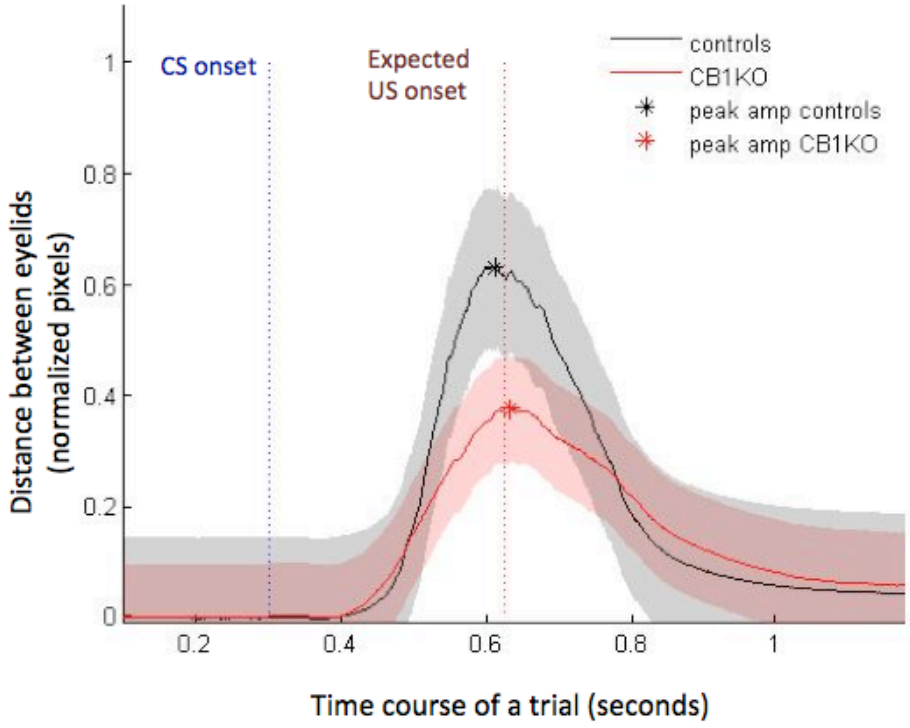

FIGURE 11.GLOBAL CB1 KO MICE - AVERAGED WAVEFORMS OF EYELID MOVEMENTS DURING CS-ONLY TRIALS AT TEST SESSIONS:

Comparison of the waveforms representing the eyelid movements during CS-only trials, in test sessions, between Global CB1 KO and control mice. The y axis represents the distance between the eyelids in normalized pixels (0 is fully opened eye, 1 is maximum eyelid closure); The x axis measures the time elapsed, in seconds, from the beginning to the end of the trials. Red line, averaged waveforms of eyelid movements in CS only trials of test sessions, in Global CB1 KO mice (CB1KO). Black line, averaged waveforms of eyelid movements in CS only trials of test sessions, in control mice. Dotted vertical lines, from left to right: timing of the CS and expected timing of US).

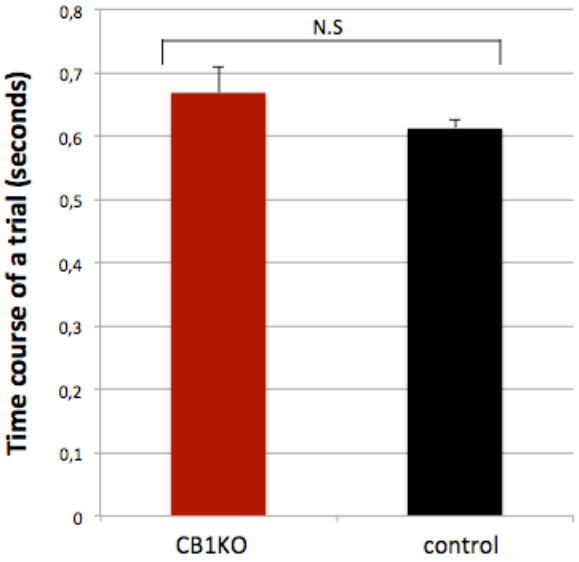

FIGURE 12.GLOBAL CB1 KO MICE - AVERAGED LATENCIES TO PEAK AMPLITUDE DURING CS-ONLY TRIALS AT TEST SESSIONS:

Comparison of the averaged latencies to peak amplitude of the CR's occurring at CS-only trials, during test sessions, between Global CB1 KO and control mice. The y axis represents time elapsed (In seconds) from the onset of a trial. The x axis represents the 2 groups being compared: Global CB1 KO (Red bar: CB1KO) and the control group (Black bar). N.S: differences are not statistically significant, p-value ≥0,05

After running experiments with Global CB1 KO mice, mice from the Alpha6 Cre CB1fx strain, that lacked CB1R's specifically from granule cells, were assessed in eyeblink conditioning. A total of 17 mice were tested (n=11 controls; and n=6 a6 CB1KO). As before, experiments consisted of 20 acquisition, 2 test and 3 extinction sessions. As an exception one mouse from the control group ran only12 acquisition sessions, followed by the test and extinction sessions, and one mouse from the a6 CB1KO group didn't do the test nor the extinction sessions.

Percentage of Conditioned Responses

The averaged percentage of CR's (%CR's) in Alpha6 Cre CB1;fx mice shows statistically significant higher values from those obtained by the control group (t-test p-value for grouped sessions, S1-S4:0,08; S5-S8:0,30; S9-S12:0,00; S13-S16:0,00) **(Fig.13** and **Fig.14)**. Despite these differences, performance of both groups reaches similar levels at the final acquisition sessions (t-test p-value for grouped sessions, S17-S20:0,25) **(Fig,13** and **Fig.14)**. These results suggest that a6CB1 mice required a higher number of sessions to develop the same level of performance as its respective controls, although they eventually catch up at later sessions (**Fig.13**). No differences were found between the 2 groups during test or extinction sessions (t-test p-values for grouped sessions T1-T2:0,43; E1-E3:0,22) (**Fig.13** and **Fig.14**).

Averaged amplitude of Conditioned Responses

Amplitude data showed decreased values for the Alpha6 Cre CB1;fx group (a6CB1 KO) when in comparison to the control group, during some of the acquisition sessions (t-test p-values for grouped sessions, S5-S8:0,55; S9-S12:0,51; S13-S16:0,04; S17-S20:0,04) **(Fig.15**

and **Fig.16)**. No differences between the 2 groups were detected during test or extinction sessions (t-test p-values for grouped sessions, T1-T2:0,99; E1-E3:0,49) **(Fig.15** and **Fig.16)**.

Averaged timing of the CR's

As observed for the global CB1 experiments, the group of mice that lack CB1R's and the control group don't show any differences in what concerns timing of the CR: both onset and offset of the response are properly timed in the KO group (a6 CB1KO) when compared to the control group (**Fig.17**). No statistically significant differences were detected for the latency to peak amplitude either (t-test p-value: 0,17) (**Fig.18**).

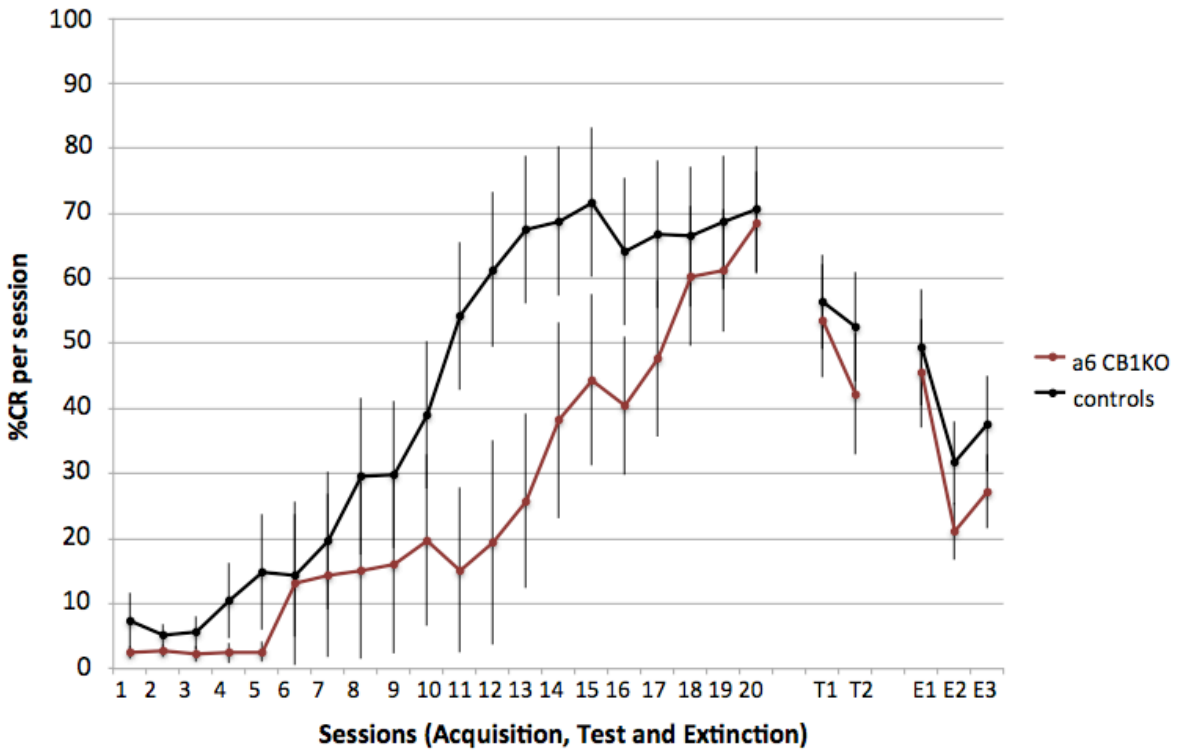

FIGURE 13.ALPHA6 CRE CB1;FX MICE - AVERAGED PERCENTAGE OF CONDITIONED RESPONSES (%CR) IN EACH SESSION:

Comparison of percentage of CR's (%CR) in Alpha6 Cre CB1;fx and control mice, throughout sessions. The y axis represents the averaged percentage of the CR's per session ; The x axis represents session number (T:Test, E:Extinction). Red line, averaged percentage of CRs Alpha6 Cre CB1;fx mice (a6 CB1KO); Black line, averaged percentage of CRs in control mice

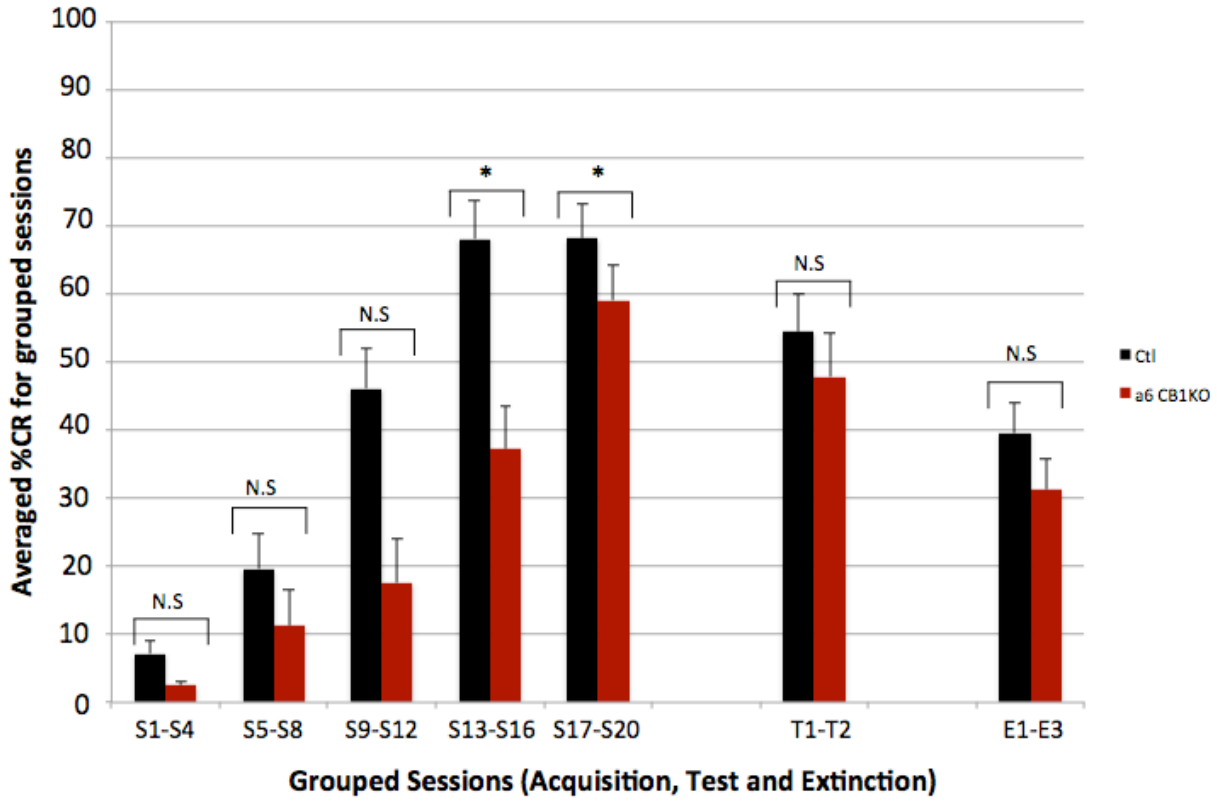

FIGURE 14.ALPHA6 CRE CB1;FX MICE - AVERAGED PERCENTAGE OF CONDITIONED RESPONSES (%CR) FOR GROUPED SESSIONS:

Comparison of percentage of CR's (%CR) in Alpha6 Cre CB1;fx and control mice in groups of 4 acquisition sessions, 2 test sessions and 3 extinction sessions . The y axis represents the averaged percentage of the CR's on each group of sessions ; The x axis represents session number (T:Test, E:Extinction). Red bars, averaged percentage of CRs in Alpha6 Cre CB1;fx mice (a6 CB1KO) for the considered interval of sessions; Black bars, averaged percentage of CRs in control mice (Ctl) for the considered interval of sessions. N.S: differences are not statisitcally significant, p-value ≥0,05; (*): statistically significant differences, p-value <0,05

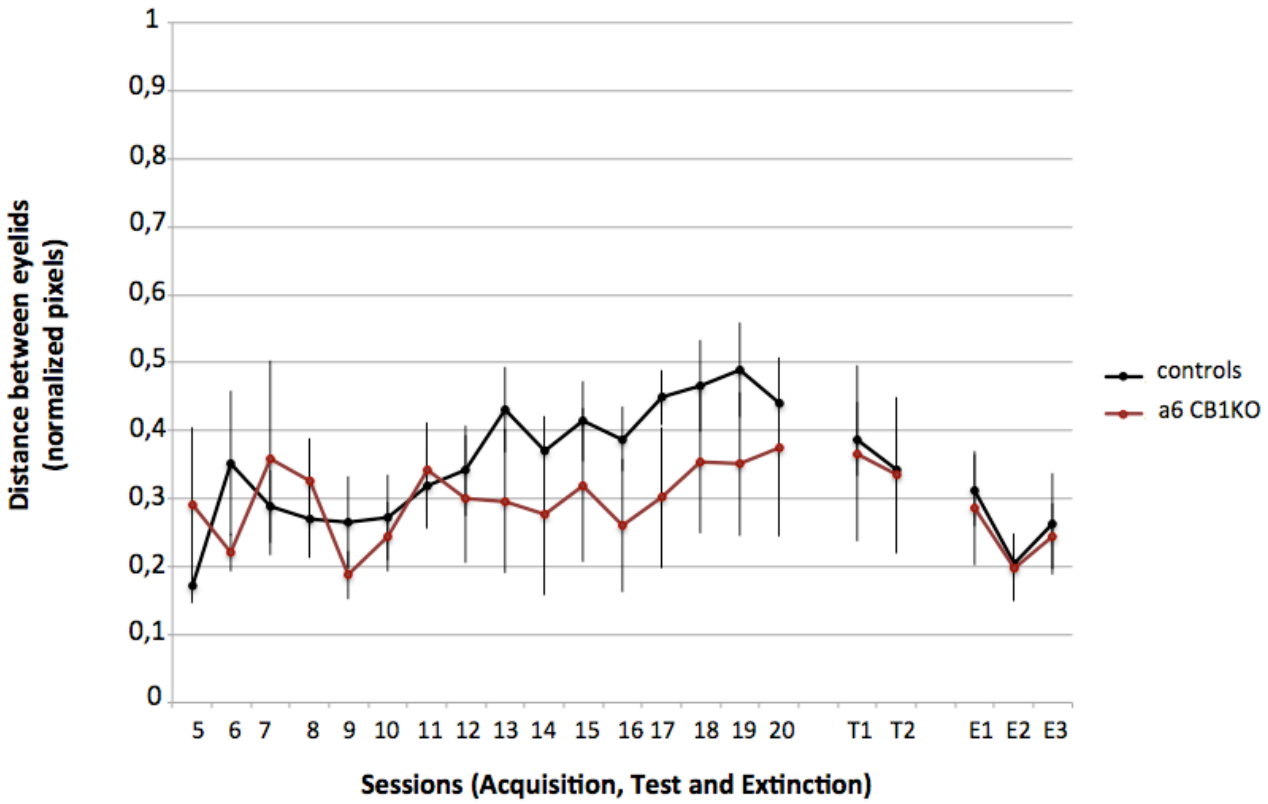

FIGURE 15.ALPHA6 CRE CB1;FX MICE - AVERAGED AMPLITUDE OF CONDITIONED RESPONSES (AVG AMP) IN EACH SESSION:

Comparison of the amplitude of the CR's (avg amp) in Global CB1 KO and control mice, from session 5 onwards. The y axis represents the normalized values of the eyelids distance in pixels (0 is fully opened eye, 1 is maximum eyelid closure) for each session; The x axis represents session number (T:Test, E:Extinction). Red line, averaged amplitudes of CR's per session, in Global CB1 KO mice (CB1KO) . Black line, averaged amplitudes of CR's per session, in control mice

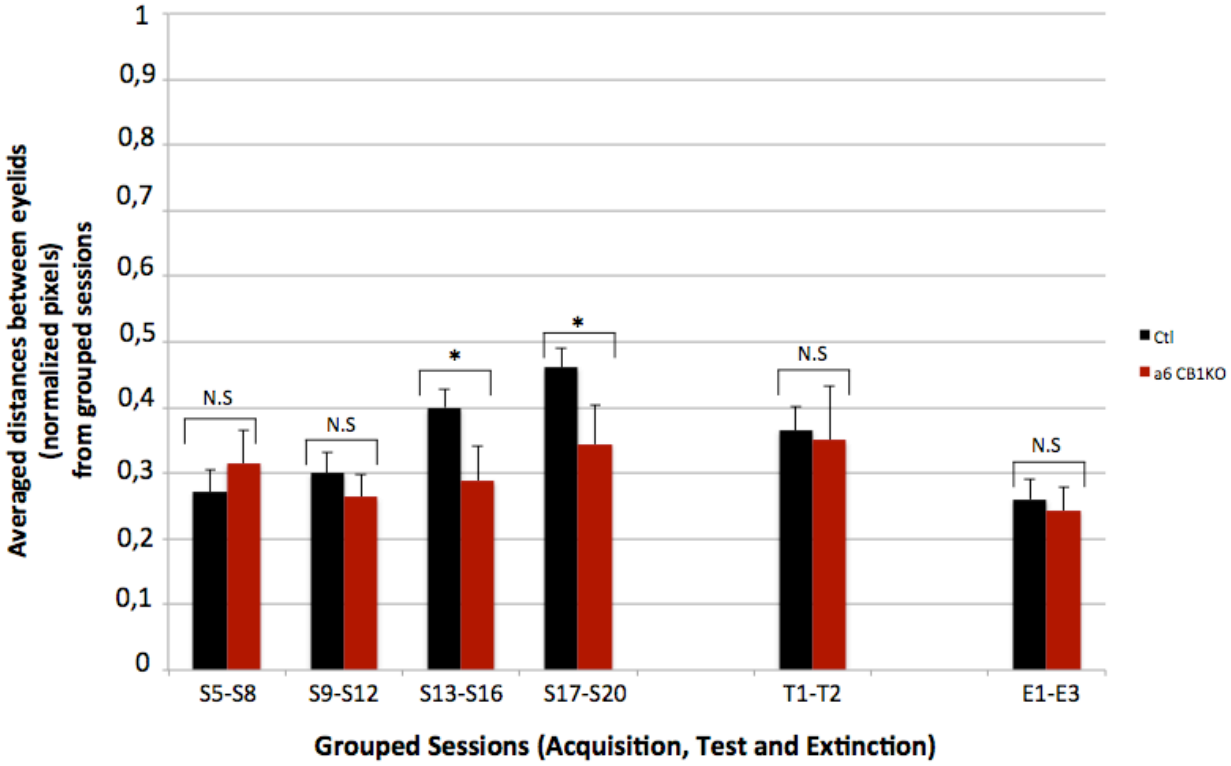

FIGURE 16.ALPHA6 CRE CB1;FX MICE - AVERAGED AMPLITUDE OF CONDITIONED RESPONSES FOR GROUPED SESSIONS:

Comparision of the amplitude of CR's in Alpha6 Cre CB1;fx and control mice in groups of 4 acquisition sessions, 2 test sessions and 3 extinction sessions . The y axis represents the averaged values of the normalized distances between eyelids, for each group of sessions ; The x axis represents session number (S:Acquisition, T:Test, E:Extinction). Red bars, averaged amplitude of CRs in Alpha6 Cre CB1;fx mice (a6 CB1KO) for the considered interval of sessions; Black bars, averaged amplitude of CRs in control mice (Ctl) for the considered interval of sessions. N.S: differences are not statisitcally significant, p-value ≥0,05; (*): statistically significant differences, p-value <0,05

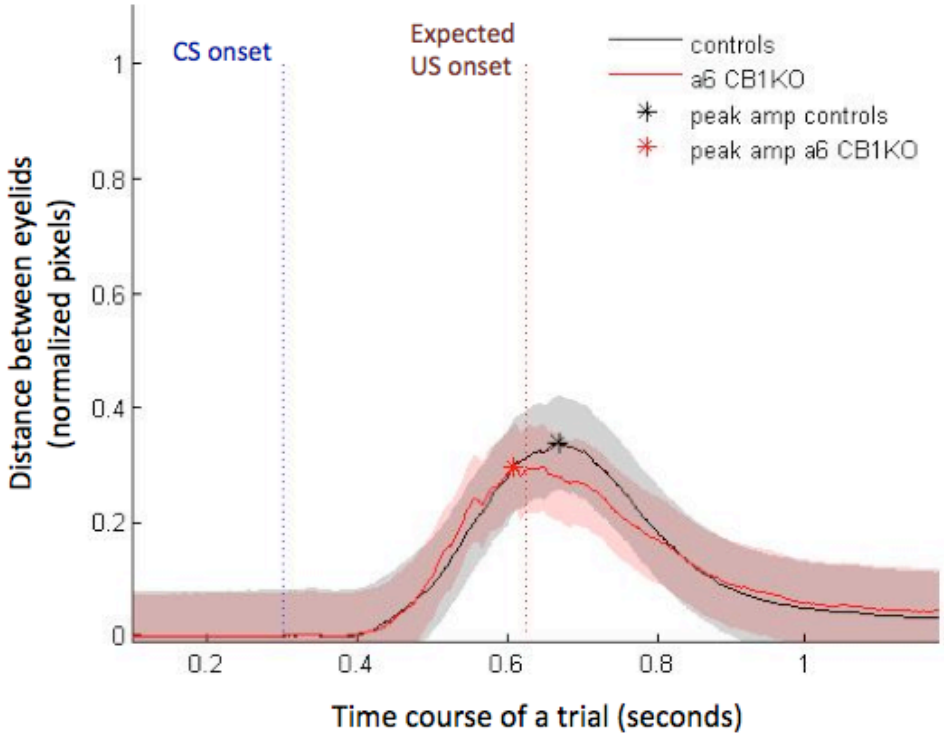

FIGURE 17.ALPHA6 CRE CB1;FX MICE - AVERAGED WAVEFORMS OF EYELID MOVEMENTS DURING CS-ONLY TRIALS AT TEST SESSIONS

Comparison of the waveforms representing the eyelid movements during CS-only trials, in test sessions, between Alpha6 Cre CB1;fx and control mice. The y axis represents the distance between the eyelids in normalized pixels (0 is fully opened eye, 1 is maximum eyelid closure); The x axis measures the time elapsed, in seconds, from the beginning to the end of the trials. Red line, averaged waveforms of eyelid movements in CS only trials of test sessions, in Alpha6 Cre CB1;fx mice (a6 CB1KO). Black line, averaged waveforms of eyelid movements in CS only trials of test sessions, in control mice. Dotted vertical lines, from left to right: timing of the CS and expected timing of US).

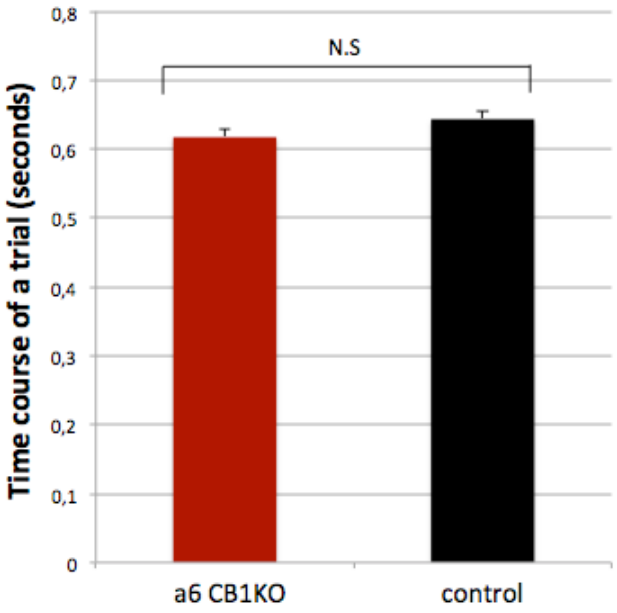

FIGURE 18.ALPHA6 CRE CB1;FX MICE - AVERAGED LATENCIES TO PEAK AMPLITUDE DURING CS-ONLY TRIALS AT TEST SESSIONS:

Comparison of the averaged latencies to peak amplitude of the CR's occurring at CS-only trials, during test sessions, between Alpha6 Cre CB1;fx and control mice. The y axis represents time elapsed (In seconds) from the onset of a trial. The x axis represents the 2 groups being compared: Alpha6 Cre CB1;fx (Red bar: a6 CB1KO) and the control group (Black bar). N.S: differences are not statisitcally significant, p-value ≥0,05

DISCUSSION

The aim of the present experimental work was to understand how, and at which cell-types, CB1Rs modulated cerebellar-dependent delay eyeblink conditioning in mice. To address these questions eyeblink conditioning was assessed by analyzing different features of the behavior - percentage, amplitude, and timing of the CR -, in both global and cell-type specific CB1 KO mice.

The results obtained here are generally in accordance with previous findings (Kishimoto & Kano 2006) , and show that the global CB1KO group (CB1KO) had significantly lower %CR during acquisition, when compared to the respective controls (**Fig.19**). However, there were also some discrepancies between the present results and Kishimoto's , which I will discuss here.

Using videography as the recording method made it possible to further analyze the amplitude and timing components of the CR. Amplitude of the CR's was also decreased in global CB1 KO mice, although no effects were observed in timing (**Fig.19**).

Alpha6 Cre;CB1 fx, a strain of mice lacking CB1R's specifically at granule cells, were assessed for the first time in eyeblink conditioning. Granule cell-specific CB1 KO mice (a6CB1 KO) also showed impairments in the %CR's expressed. However, these impairments consisted of a slower, rather than decreased expression of CR's, since performance at test sessions was indistinguishable from the control group (**Fig.19**). Although to a lesser extent than the Global CB1 KO mice, there was also some decrease in the amplitude of the CR's in granule cell-specific CB1 KO mice (**Fig.19**). However, after learning was complete, during

test sessions the granule cell specific CB1 KO group reached a performance like the controls. The CR's were also properly timed in granule cell-specific CB1 KO mice (**Fig.19**).

	Global CB1KO	Alpha6 Cre
%CR	↓	slow
Amp.	↓	⇩
Timing	OK	OK

FIGURE 19.RESUME OF RESULTS

Red Arrow down: decreased values when in comparison to the respective controls. Dashed Arrow Down: decreased values when in comparison to the respective controls, but smaller effect. OK: no differences observed, between mice lacking CB1R and the controls

Below I will discuss the current results and their implications for the role of endocannabinoids in eyeblink conditioning, and address some apparent discrepancies from previous work.

- **The % CR is decreased in global and slowed down in granule-cell specific CB1 KO mice**

Previous experiments conducted by (Kishimoto & Kano 2006) had already shown that global CB1 KO mice had decreased %CR in cerebellum-dependent eyeblink conditioning. The results obtained here confirm this finding, as global CB1 KO mice show significantly lower %CR during acquisition sessions, than the control group.

By further assessing granule cell-specific CB1 KO mice on this behavior, we also observed impairments in terms of %CR's. Importantly, in this group of mice, that lacked CB1R's specifically at granule cells, the impairment didn't consist in a decrease in %CR, but rather in a delayed acquisition of CR's: while in the global CB1 KO group the %CR at the final acquisition sessions is significantly different from that of the controls, granule cell-specific CB1 KO mice are able to reach %CR values at the final sessions comparable to those of the control group.

Although CB1R's at the Parallel fibers may be involved at some extent in the normal acquisition of the CR, the fact that stronger impairments were seen in global, rather than in granule-cell specific CB1 KO mice, suggests that some other site, where CB1R's are also expressed, is playing a major role in modulating the acquisition of CR's. This site is most likely located at the cerebellar cortex, since previous work has shown that pharmacological disconnection of the Pkj cells from the DCN prevents acquisition of CR's in naïve mice undergoing eyeblink conditioning experiments (Bao et al. 2002).

Inhibitory interneurons, like basket and stellate cells (Purves et al. 2004), seem good

candidates, as they are known to express CB1R's (Suárez et al. 2008) and they modulate Purkinje cells inhibitory inputs (Purves et al. 2004). Particularly, stellate cells, that receive connections from Parallel Fibers and in turn synapse onto Purkinje cells, could act to decrease Purkinje cells inhibitory inputs to the DCN. Furthermore, LTP has been described to occur between interneurons and the Pkj cells (Hansel et al. 2001). Stellate cells would respond to Parallel fibers signaling the CS and in turn, strengthened connections with the Purkinje cells would result in decreased inhibitory inputs to the interposed nuclei, providing the adequate level of excitability to the DCN that permits the expression of the CR.

- **Measuring Amplitude and Timing for the first time in Global and Granule-cell specific CB1 KO mice**

Instead of using Electromyography (EMG) as a recording technique, like Kishimoto and Kano did (Kishimoto & Kano 2006), a different recording method was used in the present experiments, which enabled us to analyze other components of the CR, besides the percentage: amplitude and timing. Despite being the oldest method used to record eyeblink conditioning in a variety of animals (Gruart & Blazquez 1995; Gruart et al. 2000; Kishimoto et al. 2001; Kishimoto & Kano 2006; Kotani et al. 2002; Mauk & Ruiz 1992; Trigo et al. 1999; Ivarsson & Svensson 2000), EMG has some disadvantages when compared to videography. For instance, EMG doesn't capture the actual eyelid movement, but rather the muscle activity associated to it, so it makes it harder to obtain precise spatial and temporal information about the kinematic properties of the blinks, such as position, velocity or acceleration (Koekkoek et al. 2002). Recording by means of videography, on the other hand, consists in recording the actual eyelid movements with a high-speed camera, making it possible to extract information regarding amplitude and timing of the CR's.

- **Amplitude of the CR's is decreased in Global more than in Granule cell-specific CB1 KO mice**

Amplitude data also revealed a decrease in Alpha6 Cre;CB1 fx mice, during acquisition sessions. Despite it suggests that CB1R's located at the Parallel fibers are somewhat involved in the gain of the response (this gain is expressed in terms of amplitude of the CR), stronger effects were observed in global CB1 KO mice, therefore suggesting that other sites, where CB1R's modulate plasticity, must be required for the gain of the response.

In work developed by (Kreider & Mauk 2010) where mice were trained to condition their eyelid responses to match targeted amplitudes, the authors observed that they could also learn the amplitude of the responses when the CS was substituted by direct mossy fiber stimulation, and that muscimol injections to the interpositus nucleus abolished previously acquired responses. This study provides a hypothesis for a possible site involved in the learning of amplitude: the synapse between mossy fibers and the DCN. Since CB1R's are thought to be expressed at the DCN (possibly at the interposed and lateral nuclei) (Suárez et al. 2008), it is possible that they modulate plasticity mechanisms taking place at the mossy fibers to DCN synapse, such as LTP (Hansel et al. 2001), that may underlie learning of the amplitude. In this way, and supporting a previously proposed model by (Medina & Mauk 2000), strengthened connections between MF-DCN would encode the amplitude of the CR's.

- **Conditioned Responses were properly timed in both global and granule cell specific CB1 KO mice**

Surprisingly, analysis of the onset and offset of the response, and of the latency to peak amplitude, revealed no statistically significant differences between mice lacking CB1R's and the control groups. Timing is a feature of the CR whose learning has been hypothesized to occur at the cerebellar cortex, through synaptic inputs from Purkinje cells to the DCN (Bao et al. 2002; Medina & Mauk 2000). Because no impairments in the timing of the CR were observed in global nor granule cell-specific groups, the results suggest that this component of the CR is not dependent upon CB1R's signaling.

A hypothesis could be that timing is being coded at PF-Pkj by LTP (Hansel et al. 2001). LTP at PF-Pkj has been hypothesized to suppress responses that were not required (Medina & Mauk 2000), and it could be modulating timing by selectively decreasing the strength of Purkinje cells inputs that were not appropriately timed.

Kishimoto's experimental protocol and results (Kishimoto & Kano 2006) share some features with fear based Pavlovian conditioning (Ledoux 2000; Curzon et al. 2009). Not only the type of CS and US chosen for those experiments – an auditory tone CS and a shock US-, are the most commonly utilized stimuli in fear conditioning, but also the speed of acquisition of the CR's is faster than what would be expected for a cerebellum-mediated type of learning (approx. 30% CR's in the control group, from session1). So it may be that the type of learning observed by Kishimoto and his colleagues is mediated not only by the cerebellum, but also depends upon extra-cerebellar structures such as the amygdala (Boele et al. 2010). If this is true, than one should also question whether the impairments seen in CB1 global KO mice have to do with the absence of CB1R's in the cerebellum or elsewhere in the brain - for example, in the amygdala-.

Type of CS and US

The type of CS and US chosen for the eyeblink conditioning is different depending on the animal species used and it has an important role on the outcome performance of subjects (Boele et al. 2010). The US can be either an electrical shock in the eyelid area (Kishimoto & Kano 2006; Freeman et al. 2003; Kishimoto et al. 2001; Koekkoek et al. 2002; Koekkoek et al. 2003; Kotani et al. 2002; Mauk & Ruiz 1992), or an air puff directed to the cornea of the eye (Chettih et al. 2011; Gruart & Blazquez 1995; Gruart et al. 2000; Mauk & Ruiz 1992; Schonewille et al. 2011; Trigo et al. 1999). The intensity and duration of the US also needs to be adjusted, and in the case of the air-puff its intensity may even need to be increased throughout the sessions, to prevent habituation to the stimulus (Boele et al. 2010). The CS's

that are commonly used may range from a single light stimulus (Chettih et al. 2011), a tone (Freeman et al. 2003; Gruart & Blazquez 1995; Gruart et al. 2000; Kishimoto et al. 2001; Kishimoto & Kano 2006; Koekkoek et al. 2002; Koekkoek et al. 2003; Kotani et al. 2002; Mauk & Ruiz 1992; Schonewille et al. 2011), or even an electrical shock to the forelimb (Ivarsson & Svensson 2000).

Because small differences in the CS and US and their intensities affect the subject's performance, it is important that when comparing results of the present experimental work in eyeblink conditioning with the ones previously published by other authors, these are taken into account. In most of the studies described in the literature, including (Kishimoto & Kano 2006), a tone is chosen as the CS. Here, a blue LED light CS was used for these experiments. The decision to use a light instead of an auditory tone intended to avoid the presence of an auditory startle reflex, which consists of a fast bodily response to the auditory stimulus. Importantly, the underlying circuit for this reflex is directly controlled by the amygdala, whose increased activity increases the startle reflex (Boele et al. 2010).

The stimuli used in Kishimoto's experiments were more aversive than the ones used in the present experiments (LED light *versus* auditory 80db 1Khz tone; air-puff to the cornea of the eye *versus* electrical shock to the eyelid), and they may have elicited a fear-triggered response or at least some component of this conditioned response may have been mediated by fear (Boele et al. 2010).

Speed of acquisition

Eyeblink conditioning learning typically develops gradually, on a trial by trial basis, along several sessions (Boele et al. 2010), but Kishimoto's results show a significant increase in the %CR's, compared to baseline values of spontaneous blinks, right from session 1 (Kishimoto & Kano 2006). In fact in a study conducted by (Lee & Kim 2004), where rats were tested in

delay eyeblink conditioning and fear conditioning, lesions to the amygdala resulted in a decelerated acquisition of conditioned blink responses. Furthermore, although post-training muscimol injections to the amygdala did not affect performance in eyeblink conditioning, pre-training injections led to impairment of the CR's. This can be explained by the "two-process model of conditioning": this model predicts that the first responses that emerge after only a few CS-US pairings are non-specific emotional fear responses, while the motor specific CR's require more pairings to be expressed (Boele et al. 2010). In this way, and supporting the results obtained by (Lee & Kim 2004), it is possible that these initial fear-based responses are dependent on the amygdala and are somewhat responsible for facilitating the acquisition of well-timed cerebellum-dependent responses (Boele et al. 2010).

Endocannabinoids, amygdala and Fear conditioning

Previous work by Marsicano and colleagues, showed that endocannabinoids signaling was involved in plasticity mechanisms that are thought to be underlying amygdala-mediated associative learning: long- term depression of inhibitory inputs (LTDi) to the basolateral amygdala (BLA) is abolished by CB1R antagonist SR141716A, and cannot be evoked in global CB1 KO mice (Marsicano et al. 2002; Azad et al. 2004). Although in this study the impairments observed in CB1 defective mice were observed specifically during extinction, with no alterations in acquisition or consolidation phases (Marsicano et al. 2002), another study where contextual (rather than cued) fear conditioning was tested in CB1R defective mice, described a decrease in conditioned fear responses (Mikics et al. 2006). In the same study, the use of the CB1R antagonist AM-251, also decreased the fear conditioned responses (Mikics et al. 2006).

The studies exemplified here support the hypothesis that the absence – or blocking by the use of antagonists- of CB1R's, result in deficits in the expression of conditioned responses. Since

Kishimoto's work was developed using a very similar protocol to that of cued fear conditioning (Marsicano et al. 2002; Azad et al. 2004), and considering the impairments observed by this group in CB1 defective mice, it is reasonable to assume that maybe the impaired performance of global CB1 KO in Kishimoto's paper is at some extend caused by CB1 deficits in the amygdala. Because in the results obtained in here, greater impairments in the %CR were observed for global rather than granule cell-specific CB1 KO mice, the involvement of amygdala CB1R's also cannot be ruled out.

CONCLUSION AND FUTURE DIRECTIONS

The purpose of the present experiments was to determine the role of CB1R's in cerebellum-dependent delay eyeblink conditioning, by investigating in which cell types these receptors were required for the behavior and in what way they could modulate learning.

This work was partly based on recent findings by (Kishimoto & Kano 2006) that global CB1 KO mice were impaired in delay eyeblink conditioning. In accordance with this, our results also show a decrease in the %CR during acquisition of eyeblink conditioning in Global CB1 KO mice. There are also some discrepancies between these results and the ones from (Kishimoto & Kano 2006), which could be explained by the use of more aversive CS and US in the previous work, resulting in the involvement of extra-cerebellar structures such as the amygdala.

The use of videography to record eyelid movements further enabled the analysis of other components of the CR: amplitude and timing. A strain of granule cell-specific CB1 KO mice (Alpha6 Cre;CB1 fx) was also assessed in eyeblink conditioning for the first time.

These results, together with the ones from (Kishimoto & Kano 2006), show that endocannabinoid signaling can modulate learning of the cerebellum-dependent eyeblink conditioning. Specifically, acquisition and gain of the response – measured in terms of percentage an amplitude of the CR's - were impaired in mice lacking CB1R's, both globally and specifically in the granule cells, with stronger effects on the global CB1 KO group, as expected. The fact that impairments in global CB1 KO were more severe than the ones observed for granule cell specific CB1 KO mice indicates that CB1R's at the parallel fibers cannot account alone for learning of these features. Instead, some other CB1-dependent

mechanism must be implicated. No differences in the timing of the CR were observed between mice lacking CB1R's and the respective controls, which indicates that this feature is not being modulated by CB1 signaling.

These results support the notion that different features of the eyeblink conditioning are encoded at different sites of the cerebellar circuit, and not exclusively by LTD at the PF-Pkj synapse (Carey & Lisberger 2002). In this way, further work should focus on exploring where in the cerebellar circuit are acquisition, amplitude and timing being encoded.
I hypothesize that acquisition and gain of the response could be encoded by CB1-mediated plasticity at the connections between inhibitory interneurons and the Purkinje cells, and the Mossy fiber to DCN synapse, respectively. LTP has been described in both of these synapses, and it seems a good candidate, as learning would be the result of strengthened connections at these synapses. Timing is most likely being coded at the cortical areas of the cerebellar circuit, possibly by LTP at PF-Pkj, since
To test whether acquisition and gain of the response are being coded at the aforementioned synapses, conditional CB1 KO mice lines, targeting specifically the interneurons and the interposed nuclei of the DCN, could be used to:
a) Conduct electrophysiology experiments to see if it is possible to induce LTP at these synapses, in the absence of CB1R's
b) Assess amplitude and %CR in eyeblink conditioning experiments;

Despite the present results suggesting that timing doesn't depend upon CB1R's signaling, other approaches should be used to understand where is this feature of the eyeblink condition being coded and through what mechanisms. A possible direction would be to selectively impair LTP at PF-Pkj and assess timing of the CR's in eyeblink conditioning experiments.

Importantly, and because of the low number of subjects included in the present experimental work, more experiments should be done to confirm the present results under a more representative sample.

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